Drosophila melanogaster as a model to study metal homeostasis and amyloid-ß toxicity

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Summary

Both copper and zinc are essential heavy metal elements. Their homeostasis is tightly regulated to maintain optimal concentrations for cellular functions and at the same time avoid toxic effects. The aim of my thesis was to understand how higher organisms maintain the homeostasis of these trace elements, especially Cu, in an environment with fluctuating metal availability. In a first part, using the fruit fly *Drosophila* as a model, I addressed the following questions: how is copper toxicity sensed and how does *Drosophila* protect itself against copper toxicity? To this end I used a tissue-specific reporter system in which the copper importer Ctr1B is specifically expressed in the *Drosophila* eye and causes copper overload toxicity. I found that strong expression of the metal-responsive transcription factor-1 (MTF-1), of small metal-binding proteins (metallothioneins) or of the copper transporter ATP7, the homolog of human ATP7A and ATP7B, ameliorate copper toxicity. Furthermore, in a collaboration with David Giedroc's group (Indiana University, Bloomington), we have characterized a cysteine-rich domain in *Drosophila* MTF-1 and shown that it is crucial for sensing excess intracellular copper.

The second part of my thesis focuses on how dysregulation of Zn and Cu levels might influence neurodegenerative diseases, especially Alzheimer's disease. Copper and zinc effects had been implicated in APP processing, amyloid peptide Aß aggregation, and the generation of oxidative stress. To investigate how metal homeostasis and oxidative stress affect Aß toxicity *in vivo*, we show in a transgenic *Drosophila* model that elevated Zn and Cu concentrations exacerbate Aß-induced phenotypes. By contrast, supplementation of metal chelators or strong expression of metal scavengers suppress the toxic effects of Aß. Our results support the notion that an abnormal metal homeostasis contributes to the course of Alzheimer's disease.

Zusammenfassung

Kupfer und Zink sind essentielle Spurenelemente. Ihre intrazelluläre Konzentration auf einem konstanten Niveau zu halten („Homöostase“) ist wichtig, um einerseits zelluläre Funktionen zu gewährleisten und andererseits toxischen Effekten entgegen zu wirken. Das Ziel meiner Dissertation war es, zu untersuchen wie die Homöostase dieser Spurenelemente, insbesondere von Kupfer, in höher entwickelten Organismen reguliert wird, wenn sie unterschiedlichen Metallkonzentrationen ausgesetzt sind. Der erste Teil der Arbeit befasst sich, unter Zuhilfenahme der Fruchtfliege *Drosophila* als Modellorganismus, mit folgende Fragen: Wie werden kritische Kupferkonzentrationen erkannt, und wie schützt sich die Taufliege vor Kupferüberschuss? Hierzu wurde ein gewebsspezifisches Reportersystem verwendet, bei dem der Kupferimporter Ctr1B spezifisch im Auge der Taufliegen exprimiert wird und diese dadurch Kupfer-abhängige Gewebsschädigungen im Auge aufweisen. Es zeigte sich, dass eine starke Expression des „metal-responsive transcription factor“ (MTF-1), oder der kleinen metall-bindenden Proteine Metallothioneine, oder des Kupfertransporters ATP7 (homolog zu den menschlichen Kupfertransportern ATP7A und ATP7B) den durch Kupfer verursachten Schäden entgegenwirkt. In Zusammenarbeit mit der Arbeitsgruppe von David Giedroc (Indiana University, Bloomington) haben wir zudem eine Cystein-reiche Domäne in *Drosophila* MTF-1 charakterisiert und gezeigt, dass sie als Sensor für Kupferüberschuss in der Zelle fungiert.
Im zweiten Teil meiner Arbeit ging es um die Frage, ob und wie sich eine Störung der Metall-Homöostase auf neurodegenerative Krankheiten, insbesondere die Alzheimer-Krankheit, auswirken kann. Veränderte zelluläre Kupfer- und Zinkkonzentrationen waren bereits früher mit dem Processing des Vorläuferproteins APP, der Verklumpung des Amyloid beta-Peptids (Aß) und mit oxidativem Stress in Verbindung gebracht worden. Auch hier verwendete ich *Drosophila* als Modellorganismus, indem ich ein Gen für menschliches Amyloid-Peptid ($Aß_{42}$) in das Fliegengenom integrierte. Ein Ueberschuss an Zink und Kupfer erhöhte die pathogenen Effekte von $Aß_{42}$ massiv, während die Verabreichung von kleinen metallbindenden Molekülen (“Chelatoren”) oder eine starke Expression von metallbindenden Proteinen zu einer Besserung führte. Diese Resultate deuten darauf hin, dass die zelluläre Konzentration von Zink und Kupfer den Verlauf der Alzheimerkrankheit beinflusst.

List of Important Abbreviations

AD	Alzheimer's disease
Aß	amyloid-ß
APP	amyoid-ß precursor protein
CQ	clioquinol
COX	cytochrome c oxidase
Cu-Zn SOD	Cu-Zn superoxide dismutase
MAC	membrane-activated chelator
MT	Metallothionein
MTF-1	metal-responsive transcription factor-1
MRE	metal response element
ROS	reactive oxygen species
SOD	superoxide dismutase

General Introduction

Copper homeostasis

Copper is essential for normal cell functionality due to its capacity to shift between two transition states, Cu(I) and Cu(II). It is present within redox enzymes such as Cu/Zn-superoxide dismutase (Cu/Zn SOD), tyrosinase, and cytochrome c oxidase (COX). The activity of these enzymes is dependent on optimal copper binding. Thus, eukaryotic organisms from yeast to humans use elaborate systems to regulate copper homeostasis (O'Halloran and Culotta, 2000; Puig and Thiele, 2002; Mercer and Llanos, 2003; Balamurugan and Schaffner, 2006). Copper deficiency prevents the normal activity of these cuproenzymes and leads to cell damage. For example, under copper deficiency, oxidative stress increases due to less Cu-Zn SOD activity. In humans, mutations in the copper transporter ATP7A gene lead to decreased supply of copper and cause Menkes disease. It has also been implicated that Alzheimer's disease (AD) patients show copper deficiency in their brains. On the other hand, when copper accumulates freely in the cell, it contributes directly to the production of reactive oxygen species (ROS) via the Fenton reaction, resulting in oxidative damage to DNA, proteins and lipids (Halliwell and Gutteridge, 1990; Puig and Thiele, 2002). In Wilson disease patients, due to the mutations in the copper transporter ATP7B gene, copper accumulates in the liver and the brain, causing liver cirrhosis and neurological symptoms. In dogs, mutation of the COMMD1/murr1 gene, which may lead to stronger interaction of COMMD1 with ATP7B, is associated with compromised copper transport and copper accumulation, causing liver cirrhosis similar to Wilson disease. XIAP (X-linked inhibitor of apoptosis protein) is another Cu-associated protein. When it binds copper it loses its activity, which results in apoptotic cell death.

Copper is an essential trace metal, yet toxic when in excess. Therefore, cells have developed sophisticated systems to control copper levels. The concentration of unbound copper within the cell is extremely low. In eukaryotes, copper is imported as Cu(I) by high-affinity copper transporters of the Ctr family. Ctr importers form a homotrimeric complex in the membrane and acquire copper in an ATP independent manner. In yeast, three Ctr family members have been described, namely, yCtr1, yCtr2 and yCtr3. Both yCtr1 and yCtr3 locate to the plasma

membrane and import extracellular copper into the cell (Dancis et al, 1994; Pena et al, 2000). Excess copper is stored in the vacuole. yCtr2, which is localized in the vacuolar membrane, imports copper from the vacuolar storage site into the cytoplasm upon copper depletion (Rees et al., 2004). Humans have two Ctr proteins, hCtr1 and hCtr2. hCtr1 has been shown to be main copper importer, while the function of hCtr2 is not clearly understood so far (Lee et al., 2002; Zhou and Gitschier, 1997). *Drosophila* contains three Ctr family members, designated Ctr1A, Ctr1B and Ctr1C (Zhou et al., 2003). Ctr1A is constitutively expressed from four-hour-old embryos to adults and a Ctr1A null mutation causes embryonic lethality. Ctr1B expression is more abundant in late embryonic stages and in larvae than in early embryonic stages and in adults. Of note, the Ctr1B gene is transcriptionally up-regulated upon copper starvation (Selvaraj et al., 2005). Interestingly, the expression of Ctr1C is restricted to late larvae and male adults, where it contributes to male fertility (M. Fetchko, A. Vardanyan, D. Steiger and W. Schaffner, unpublished). Besides the Ctr importers, copper can also be acquired via less specific, low affinity metal transporters, such as DMT1 (divalent metal transporter 1). However, studies in yeast, *Drosophila* and mouse models have shown that such an acquisition mechanism is not sufficient to substitute for the function of Ctr proteins (Valentine et al., 1997; Zhou and Gitschier, 1997; Lee et al., 2002; Huang et al., 2002; Puig and Thiele, 2002; Van Ho et al., 2002; Zhou et al., 2003; Petris 2004; Selvaraj et al., 2005). There is recent evidence that copper may be acquired by the intestinal cells via membrane internalization.

Upon its import into the cell, Ctr transporters deliver copper to specific chaperones. There are three groups of copper chaperons that accept copper from Ctr proteins and transfer it to their target proteins. CCS delivers copper to Cu/Zn SOD (Wong et al., 2000; Schmidt et al., 2000); Cox17 transfers copper to mitochondrial cytochrome c oxidase (Horng et al., 2004). In yeast, Atx1 brings copper to Ccc2, a copper transporting ATPase (Arnesano et al., 2001). In human, Atox1, the homolog of yeast Atx1, delivers copper to ATP7A and ATP7B proteins which localize to the trans-Golgi network (Walker et al., 2002). ATP7A, also called Menkes disease protein, transports copper from intestinal cells into the blood. ATP7A also directs copper into several cuproenzymes including tyrosinase (Camakaris et al., 1999). The related ATP7B, also called Wilson disease protein, assembles copper into ceruloplasmin, a serum

ferroxidase (Hellman et al., 2002). As mentioned, *Drosophila* contains an ortholog of mammalian ATP7A and ATP7B genes, designated DmATP7. DmATP is important in delivering copper to cuproenzymes such as tyrosinase and in the removal of excess celluar copper (Norgate et al., 2006).

When copper is in excess, Ctr importers are down-regulated transcriptionally (Ctr1B in *Drosophila*) or posttranscriptionally (e.g., hCtr1 in human) (Petris et al., 2003; Selvaraj et al., 2005). As an early response to copper load, ATP7A moves from the trans-Golgi network to the plasma membrane and pumps out excess copper. As soon as the copper level falls below a certain threshold, ATP7A moves back to the trans-Golgi network (Gitlin, et al., 1999; Voskoboinik and Camakaris, 2002). ATP7B protein is necessary to excrete copper from the liver into the bile (Oude Elferink and Groen, 2002). However, a sudden increase in copper concentration still poses a serious challenge. For that, cells rely on a metal sequestration mechanism, which is mainly performed by a group of small, cysteine-rich proteins called metallothioneins (MTs) (Kägi et al., 1991; Palmiter, 1998). Metallothioneins exists in all eukaryotes and have a high capacity to chelate zinc, copper and cadmium. There are two MT genes in budding yeast *Saccharomyces cerevisiae*, *cup1* and *crs5* (Ecker et al., 1986; Culotta et al., 1994). It has been shown that Cup1 plays a major role in copper detoxification (Jensen et al., 1996,). *Drosophila melanogaster* contains five MT genes, namely, MtnA-E (Egli et al., 2003). MtnA and MtnB are the major metal scavengers, whereby MtnA has a preference to sequester copper and MtnB has a preference for cadmium (Egli et al., 2006a). MtnC and MtnD have probably arisen by duplication events from MtnB and have a lesser role in metal sequestration (Egli et al., 2006b). MtnE, also related to MtnB, is a newly discovered metallothionein gene (D. MacAlpine and D. Thiele, personal communication). C. elegans has two MT genes, mtl-1 and mtl-2. There are four metallothioneins in the mouse (MT1 to MT4) and at least 12 (plus several pseudogenes) in human. Besides their function as metal scavengers, metallothioneins may also serve as metal storage proteins, and thanks to the abundance of thiol groups are able to scavenge radicals.

Zinc homeostasis

Zinc is an essential heavy metal that functions as a structural component in a great number of proteins, including transcription factors and enzymes. Thus it is not surprising that zinc is important for many biological processes, such as nucleic acid metabolism, cell growth and proliferation, immune response and brain development. Zinc concentrations in the cell are tightly regulated. Zinc ions are charged and cannot cross the cell membrane by passive diffusion. Hence, uptake and efflux of zinc require a set of proteins called zinc transporters. Zinc transporters are grouped into two families based on their structure and functions. The ZIP protein family (also called ZRT/IRT-related proteins) import zinc from extracelluar space or intracellular vesicles into the cytoplasm (Eide, 2004). The ZnT (zinc transporter) proteins are involved in intracellular traffic and excretion of zinc (Liuzzi et al., 2004; Palmiter and Huang, 2004). In mammals, ZIPs are encoded by solute carrier family 39A (SLC39A) genes and ZnTs are encoded by solute carrier family 30A (SLC30A) genes. Mice lacking ZIP1, ZIP2 or ZIP3 are embryonic lethal under zinc-limiting conditions (Dufner-Beattie et al., 2006; Peters et al., 2007). It has been found that certain mutations in human ZIP4 cause a zinc depletion disorder termed acrodermatitis enteropathica (AE) (Wang et al., 2002). Homozygous ZIP4 mutant mice die in early embryonic stages while heterozygous mutants are very sensitive to zinc deficiency, similar to AE patients. Furthermore, female mice lacking functional ZnT4, which is expressed in breast epithelial cells, have low zinc levels in the milk resulting in the death of the pups (Huang and Gitschier, 1997). In *Drosophila*, two ZIP genes have been identified, termed *fear of intimacy (foi)*, the counterpart of mammalian ZIP6/ZIP10 and *catsup*, the counterpart of mammalian ZIP7 (Stathakis et al., 1999; Mathews et al., 2005). One ZnT protein, designated ZnT35C, has been described in *Drosophila*. Similar to copper, intracellular zinc is buffered by metallothioneins (Yepiskoposyan et al., 2006). While *Drosophila* MTs preferentially bind copper and cadmium, mammalian MTs predominantly bind zinc, at least in non-metal-stressed cells (Palmiter, 2004).

Metal-responsive transcription factor-1 (MTF-1)

In response to zinc, copper, and cadmium, metal-responsive transcription factor-1 (MTF-1) actives cellular defense mechanisms (Westin and Schaffner, 1988; Lichtlen and Schaffner, 2001). MTF-1 is a zinc-finger transcription factor and its DNA binding domain consists of six C2H2 zinc fingers (Radtke et al., 1993; Dalton et al., 1997). Therefore, zinc is essential for the DNA binding affinity of MTF-1. The cognate DNA binding site of MTF-1 is termed metal-response element (MRE, core consensus TGCRCNC) (Stuart et al., 1984; Stuart et al., 1985). Since copper and cadmium cannot substitute for zinc in DNA binding, they regulate MTF-1 indirectly by displacing zinc from zinc-loaded MTs or possibly other zinc binding proteins (Bittel et al., 1998; Zhang et al., 2003). Such a mode of action has been shown in an *in vitro* system (Zhang et al., 2003). However, MTF-1 is subject to more complex regulation *in vivo* including phosphorylation and nuclear translocation (Saydam et al., 2001; Saydam et al., 2002). Human MTF-1 harbors a non-canonical nuclear localization signal (NLS) sequence overlapping with the zinc finger domain (U. Lindert, M. Cramer, M. Meuli, O. Georgiev and W. Schaffner, unpublished) and a nuclear export signal (NES) sequence (LCLSDLSLL) that overlaps with the major activation domain (Saydam et al., 2001). MTF-1 contains three different transactivation domains C-terminal to the DNA binding domain: an acidic, a proline-rich, and a serine/threonine-rich domain. MTF-1 is mainly cytoplasmic under normal situations but is translocated to the nucleus upon metal load or other stress conditions. When MTF-1 accumulates in the nucleus, it binds to the MREs and actives its target genes including metallothioneins, ZnT1 and placenta growth factor (PIGF) (Langmade et al., 2000; Cramer et al., 2005). MTF-1 has been identified from human, mouse, pufferfish *Fugu rubripes*, zebrafish *Danio rerio*, *Drosophila* and capybara (*Hydrochoerus hydrochaeris*). MTF-1 is well conserved among vertebrates, but less so between mammals and insects; the similarity between human and *Drosophila* MTF-1 (dMTF-1) is restricted to the zinc finger region. MTF-1 knockout mice die at the embryonic stage due to liver degeneration (Gunes et al., 1998). *Drosophila* MTF-1 null mutants are viable but highly sensitive to heavy metal stress and, interestingly, also to copper starvation (Egli et al., 2003). Further studies revealed that *Drosophila* MTF-1 (dMTF-1) has the unique ability to handle both extremes, namely, copper overload and copper depletion (Selvaraj et al.,

2005). Upon copper stress, it activates metallothionein genes, while at low copper condition it up-regulates the copper importer Ctr1B. Recently, it was also found that Ctr1B is activated by dMTF-1 as a protective mechanism of the fruit flies against cadmium or mercury toxicity (Balamurugan et al., in press).

Metals, oxidative stress and Alzheimer's disease pathology

Alzheimer's disease (AD) is a progressive brain disorder that is characterized by amyloid plaques and neurofibrillary tangles in the brain. AD patients lose their memory, cognitive functions and independence, resulting in enormous economic and social cost. Until now there is no cure for Alzheimer's disease and its molecular basis is only partially understood. The deposition of aggregated amyloid ß (Aß) peptide and tau protein, the major components of amyloid plaques and neurofibrillary tangles, respectively, have been shown to contribute substantially to the neuronal damage and pathology of AD (Goedert and Spillantini, 2006). Aß is produced via proteolytic cleavage of a type I transmembrane protein called ß-amyloid precursor protein (APP). Aß peptides occur in various lengths (35-49aa) with Aß40 and Aß42 being the predominant species. Aß42 is more aggregation-prone than Aß40 and therefore its abundance is best correlated with the disease (Hilbich et al.,1991).
A distortion in copper homeostasis is evident in the brains of AD patients. Copper is concentrated in the amyloid plaques and cerebral extracelluar copper levels in the AD-affected brain are considerably higher than in age-matched healthy brains (Lovell et al., 1998). On the other hand, there is a deficiency in intracellular copper, indicated by the decreased activities of several cuproenzymes (e.g. Cu/Zn-SOD, COX) (Maurer et al., 2000; Cottrell et al., 2001). In a transgenic mouse model of AD the loss of Cu/Zn-SOD activity could be restored by dietary copper supplementation (Bayer et al., 2003). Both APP and Aß bind Cu. Overexpression of APP showed decreased intracellular Cu levels in the brain of transgenic mice. Copper scarcity may indirectly contribute to the increased oxidative stress by lowering the activity of Cu/Zn SOD. Aß binds Cu via three N-terminal histidine residues. *In vitro* studies showed that Cu promotes oligomerization of Aß, especially at low pH (Atwood et al., 1998). Besides that, interactions between Cu and Aß result in H_2O_2 and free radical generation (Huang et al., 1999). H_2O_2 and the hydroxyl radical OH• are neurotoxic via reactions with lipids, proteins and nucleic

acids that lead to extensive damage to the cell. Furthermore, oxidation of Aß by OH• increases Aß aggregation through a di-tyrosine mediated crosslinking. Similar to Cu, homeostasis of Zn is disturbed in the AD brain. Amyloid plaques contain high levels of Zn. However, by contrast to Cu, Zn is not redox-active and does not contribute directly to oxidative damage. Zn is important for normal brain function, especially at the glutamatergic synapses. Zn is loaded into synaptic vesicles by the zinc transporter ZnT3 and released into the synaptic cleft during neurotransmission (Palmiter et al., 1996). Within the synaptic cleft, zinc concentration can reach approximately 300 μM. In a transgenic model for AD, mice lacking functional ZnT3 exhibited a 50% decrease in amyloid plaque burden compared to ZnT3 proficient littermates (Lee et al., 2002). At physiological concentration range, Zn strongly induces the aggregation and fibril formation of Aß via direct binding (Bush et al., 1994). In a cell free system, metal chelators such as EDTA can inhibit Cu- or Zn-triggered oligomerization of Aß (Bush et al., 1994). In a study with a mouse model, a moderate metal chelator clioquinol (CQ) was able to cross the blood-brain barrier and to significantly reduce amyloid plaque formation, presumably by stripping metals away from Aß peptides (Cherny et al., 2001). A more recent study demonstrated another possible mechanism for the beneficial effects of CQ, namely, it can function as a Cu carrier and increase Cu bioavailability in the cell, thus in fact counteracting Cu deficiency (Treiber et al., 2004). Whatever the mechanism, this promising result demonstrates that by directly targeting Aß peptide, modulating exctracellular and intracellular metal levels and reducing oxidative stress may be potential therapeutical strategies.

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Results

Publications

Copper homeostasis in Drosophila by complex interplay of import, storage and behavioral avoidance. (Balamurugan et al., 2007)

This publication reports a comprehensive study on how an organism, *Drosophila*, handles copper at different levels. For the first time, this publication shows that the copper importer Ctr1B protein persists at high copper concentrations while the gene has been downregulated. My part in this publication was showing that *Drosophila* seems to risk excessive copper accumulation for the potential benefit of copper donation to the next generation. I also showed in an eye expression system that metallothioneins and the copper exporter DmATP7 counteract copper toxicity.

Copper sensing function of Drosophila metal-responsive transcription factor-1 is mediated by a tetranuclear Cu(I) cluster. (Chen*, Hua*, Balamurugan*, et al., 2008) (* co-first authors)

This publication reports a novel cysteine-rich domain in *Drosophila* MTF-1. It is the first study showing how MTF-1 directly senses intracellular copper levels. My contribution to this paper was the analysis of the *in vivo* function of the cysteine-rich domain in *Drosophila* MTF-1.

Mercury and cadmium trigger expression of the copper importer Ctr1B, which enables *Drosophila* to thrive on heavy metal-loaded food. (Balamurugan et al., 2009)

This publication explored a seemingly fortuitous phenomenon, namely, that cadmium and mercury strongly activate the expression of a Ctr1B copper importer transgene. We demonstrated that copper plays a key role in protecting *Drosophila* against cadmium and mercury load and that mutant flies lacking Ctr1B are extremely sensitive to cadmium and mercury treatment. I contributed to the analysis of Ctr1B mutant flies and the preparation of the manuscript.

Effects of metal homeostasis and oxidative stress on Aß42 induced apoptosis in a Drosophila model for Alzheimer's disease.

This manuscript describes a *Drosophila* model of Alzheimer's disease. The study is focused on how different factors (e.g. metals, oxidative stress) influence the toxicity of Aß in a living organism. Expression of Aß42 peptides in *Drosophila* leads to phenotypes such cell death, tissue degeneration and defects in locomotion activity. High levels of zinc and copper exaggerate Aß42 induced phenotypes while copper/zinc chelators and the expression of MTF-1, metallothioneins and anti-oxidant genes such as glutamate-cysteine ligase, are able to reduce the toxicity of Aß42.

Discussion and Outlook

Copper homeostasis via complex cellular and behavioral responses

The intracellular level of copper, an essential trance element, is tightly regulated to ensure its essential functions and at the same time to avoid its toxicity. In *Drosophila*, expression of the Ctr1B copper importer is regulated by the transcription factor MTF-1. MTF-1 activates the Ctr1B gene upon copper scarcity and downregulates it when copper concentration is high. However, our recent results imply that there is no posttranscriptional regulation of Ctr1B in *Drosophila* and high copper levels in the food or in the cell do not lead to degradation or endocytosis of Ctr1B. This results in the accumulation of copper, which is advantageous when flies thereafter encounter a period of copper scarcity. We have shown that stored copper is transferred to the next generation such that the offspring can survive on food lacking a sufficient amount of copper. Interestingly, we find that not only females but also male parents contribute to this trans-generation effect (Fig 1). This is consistent with the fact that copper transporters are crucial for male and female fertility in *Drosophila* (M. Fetchko, A. Vardanyan, D. Steiger and W. Schaffner, unpublished). Another interesting phenomenon observed in our lab is that a slowed-down development may help the flies to mitigate copper toxicity and to survive in an environment with high levels of copper ions.

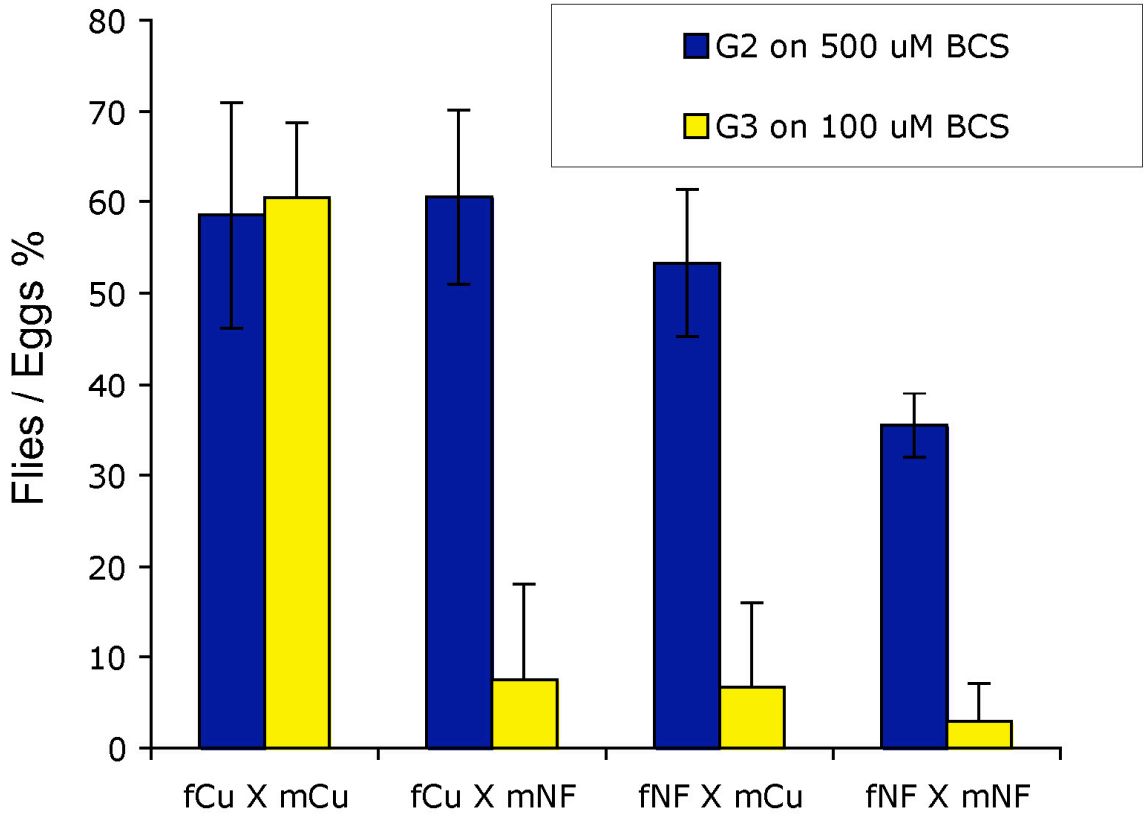

Figure 1: Copper supply to following generation

Drosophila larvae of generation 2 (G2) thrive on copper-starved food if their parents (fCu X mCu) had been raised during larval stage in copper-supplemented food. Controls whose parents grew up in standard food (fNF X mNF) are severely delayed (not shown), and many fail to develop to adulthood, as illustrated by the ratio of flies that eclosed from originally laid eggs on 500 μM of copper chelator (BCS). The beneficial effect of copper transfer to G2 even extends to a further generation (G3) raised again in low-copper food (100 μM BCS). Both male and female G1 parents are responsible for the trans-generation copper supply effect because when either male or female parents were grown on NF, their G3 generation exhibited a decreased survival rate (fCu X mNF and fNF X mCu).

Sensing of different metals by MTF-1

Metal-responsive transcription factor-1 (MTF-1) plays a crucial role in sensing metal stress (e.g. zinc, copper and cadmium toxicity) and other stress conditions and activating genes for detoxification. Although MTF-1 upregulates its target genes via the same DNA sequence termed metal response element (MRE), different subsets of the target genes are activated upon different stress conditions. For example, in *Drosophila*, MTF-1 activates metallothioneins upon copper load but upregulates the copper importer Ctr1B under copper scarcity. Also in *Drosophila,* MTF-1 activates the transcription of distinct metallothionein genes (MtnA-D) in response to Cu(I) and Cd(II). Deletion of specific metallothionein genes reveals that MtnA and MtnB are particularly suited for detoxification of Cu or Cd, respectively. The expression of the zinc efflux transporter ZnT35C is induced by MTF-1 when the flies are treated with food containing high levels of Zn. How MTF-1 protein senses a particular metal ion or a particular stress condition is still under investigation. It is clear that the sensing mechanism contains multiple levels of regulation. Zn(II) binding to the zinc-finger domain is at least part of the zinc sensing mechanism (reference). G. Andrews and colleagues provided evidence that zinc sensing of mouse MTF-1 involves the linker peptides between the zinc fingers (reference). On the other hand, MTF-1 is capable of forming a complex with Cu(I) via a C-terminal cysteine-rich domain. This domain contains six closely spaced cysteines that form a polydentate [Cu4-S6] cage structure. This domain is crucial for MTF-1 to up-regulate MtnA upon copper overload. By contrast, this cysteine-cluster is not necessary for MTF-1 to active Ctr1B upon copper starvation. Interestingly, the cysteine-cluster also mediates the response to cadmium, but not to zinc (Fig 2, 3). The sensing of Cd(II) may also involve other MTF-1 domains because flies with a mutant form of the cysteine-cluster are still able to mitigate low levels of cadmium and only display sensitivity to high levels of cadmium. Accordingly, MTF-1 that contains cysteine mutations also partly induces MtnA upon treatment with cadmium. In fact, the cysteine-cluster in dMTF-1 is able to form complexes with both Cd(II) and Zn(II), but Cu(I) easily outcompetes Zn(II) and Cd(II). In conclusion, the cysteine-rich domain is necessary for MTF-1 to sense Cu(I) and also contributes to the cadmium response.

The sensing of Zn(II) overload and copper scarcity must be mediated by hitherto less characterized domains.

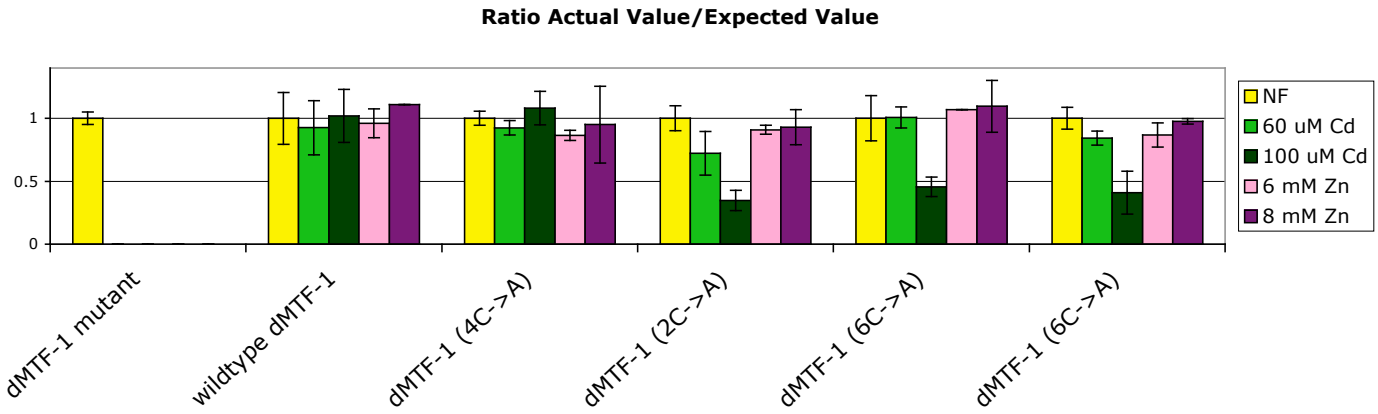

Figure 2: The cysteine-rich region helps to protect *Drosophila* from cadmium toxicity but is dispensable in sensing zinc

Shown is the survival of dMTF-1 null flies and flies expressing dMTF-1$^{2C\text{-}2A}$, dMTF-1$^{4C\text{-}4A}$ or dMTF-1$^{6C\text{-}6A}$, encoding double (C560A/C565A), quadruple (C547A/C549A/C552A/C554A) or complete (C547A/C549A/C552A/C554A/C560A/C565A) alanine substitutions, on a standard food source (NF), or on food supplemented with 50 or 100 μM $CdCl_2$ (Cd) or 6 and 8 mM $ZnCl_2$ (Zn).

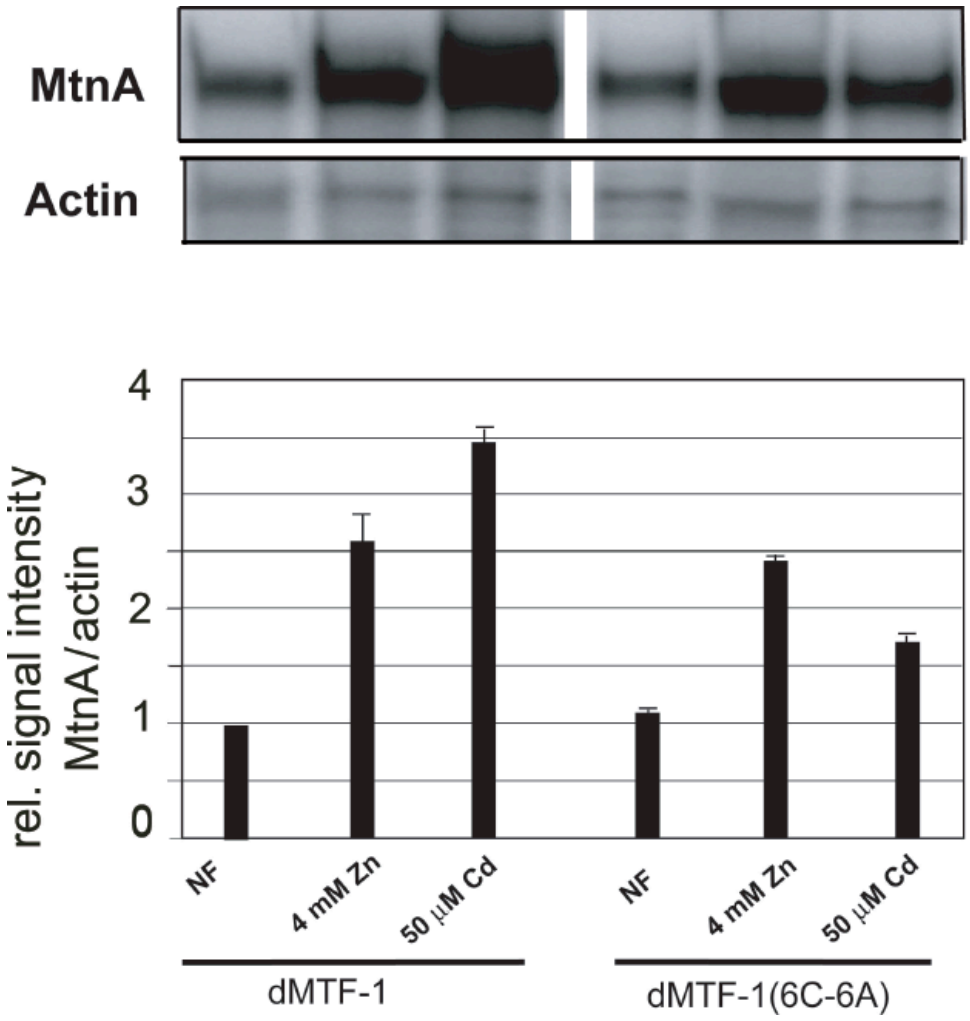

Figure 3: The Cys-rich domain of dMTF-1 is required to activate MtnA expression upon Cd, but not Zn, overload in transgenic flies

(A) Total RNA was isolated from transgenic Drosophila at the third instar larval stage expressing either a wild-type dMTF-1, dMTF-1$^{4A\text{-}4A}$, dMTF-1$^{2C\text{-}2A}$ or dMTF-1$^{6C\text{-}6A}$ allele raised on normal food (NF) or on 4 mM $ZnCl_2$ or 50 μM $CdCl_2$.

Modulation of metal bio-availability as a potential therapeutic approach for Alzheimer's disease

The Alzheimer's disease-associated amyloid peptide Aß is readily aggregated and precipitated by addition of Zn(II) and Cu(II) in the nanomolar range. This could at least in part explain why the amyloid deposits are typically associated with synapses, even though the expression of Aß is ubiquitious. The micromolar concentrations of Zn(II) and Cu(II) that are released from synapses are sufficient to trigger Aß aggregation. This may also explain why amyloid deposition is age-dependent, since loss of metal homeostasis is an important feature of the aged brain. The effects of Zn and Cu on Aß go beyond amyloid formation. Interacting with Cu, Aß generates H_2O_2 and free radicals. Human Aß42, which is most critical for the development of AD, displays a stronger redox activity (H_2O_2 and OH• formation) than Aß40. Rodent (mouse or rat) Aß peptides, which show decreased metal interaction, have almost no redox activity. AD pathology leads to dysregulation of metal levels. The observed increase of Zn and Cu in the extracelluar compartments correlated with decreased intracellular Zn and Cu levels in the brain of AD patients. There have been several attempts to develop therapies based on metal-complexing small molecules. The general metal chelator desferrioxamine, which binds Al, Zn, Cu, and Fe, gave beneficial effects in AD patients in a clinical trial. The lipophilic chelator DP109, which is more selective to Cu and Zn, reduced amyloid pathology in a transgenic mouse model of AD. Another Cu/Zn chelator, clioquinol (CQ), could reduce amyloid deposition in transgenic mice and increase Zn and Cu levels in the treated mice. In a Phase II clinical trial, CQ reduced the cognitive decline and lowered the plasma Aß levels comparing to the placebo controls. A derivative of CQ, PBT2, which is better tolerated and shows improved BBB penetration, is subject to a Phase II clinic trial. Although cliquinol in vitro can disaggregate metal containing Aß amyloid and may reduce Aß toxicity by inhibiting the redox reactions, its mechanism of action may not simply involve the chelation of Zn and Cu. CQ treatment increased Zn and Cu levels in the brain of transgenic mice and in the plasma of AD patients. In a cell culture system, CQ-Cu complexes entered APP-overexpressing cells and restored Cu/Zn SOD activity caused by Cu deficiency. Hence therapeutic approaches that modulate metal bio-availability may ameliorate

Alzheimer's disease by binding of extracelluar metals, thereby reducing Aß toxicity and, by delivering the metals into the cells, restore the activity of metalloenzymes. Such metal-binding small molecules could be combined with other potential therapies that target APP processing (secretase inhibitors) or Aß clearance (immunotherapy).

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Copper homeostasis in *Drosophila* by complex interplay of import, storage and behavioral avoidance

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Copper is an essential but potentially toxic trace element. In *Drosophila*, the metal-responsive transcription factor (MTF-1) plays a dual role in copper homeostasis: at limiting copper concentrations, it induces the Ctr1B copper importer gene, whereas at high copper concentrations, it mainly induces the metallothionein genes. Here we find that, despite the downregulation of the Ctr1B gene at high copper concentrations, the protein persists on the plasma membrane of intestinal cells for many hours and thereby fills the intracellular copper stores. *Drosophila* may risk excessive copper accumulation for the potential benefit of overcoming a period of copper scarcity. Indeed, we find that copper-enriched flies donate a vital supply to their offspring, allowing the following generation to thrive on low-copper food. We also describe two additional modes of copper handling: behavioral avoidance of food containing high ($\geqslant$0.5 mM) copper levels, as well as the ability of DmATP7, the *Drosophila* homolog of Wilson/Menkes disease copper exporters, to counteract copper toxicity. Regulated import, storage, export, and avoidance of high-copper food establish an adequate copper homeostasis under variable environmental conditions.

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Introduction

Copper is an essential component of several important enzymes involved in respiration, oxidative stress protection, pigmentation and iron homeostasis. Most of these enzymatic reactions rely on the ability of copper to undergo redox transitions between the Cu(I) and Cu(II) state. This important trait of copper is at the same time a threat to the organism, as copper can catalyze the generation of reactive oxygen species (ROS) via the Fenton reaction (Halliwell and Gutteridge, 1990; Puig and Thiele, 2002). Eukaryotic organisms from yeast to humans use elaborate systems to regulate copper homeostasis, consisting of copper importers, copper chaperones, transcription factors, small metal binding proteins called metallothioneins and copper exporters (O'Halloran and Culotta, 2000; Puig and Thiele, 2002; Mercer and Llanos, 2003; Balamurugan and Schaffner, 2006). Studies in yeast have identified the high-affinity copper transporters yCtr1 and yCtr3 (Dancis *et al*, 1994; Pena *et al*, 2000). Homologs of these were identified in mammals (Ctr1) and in *Drosophila* (Ctr1A, B and C) (Lee *et al*, 2000; Zhou *et al*, 2003). A null mutant of *Ctr1* in the mouse is lethal, whereas a mutation of *Ctr1B* in *Drosophila* results in a pigmentation defect and lethality under conditions of copper scarcity (Kuo *et al*, 2001; Lee *et al*, 2001; Zhou *et al*, 2003).

The low-copper phenotype in the fly is reminiscent of the phenotype of a mutation in the metal-responsive transcription factor-1 (MTF-1) (Egli *et al*, 2003). We have recently shown that *Ctr1B* transcription is activated by dMTF-1 under normal conditions and to a greater extent upon copper depletion, but repressed when copper is abundant (Selvaraj *et al*, 2005). In contrast, when copper is in excess, MTF-1 activates transcription of the genes for metallothioneins, small, cysteine-rich metal scavenger proteins. The genes for *Ctr1B* and the metallothioneins are therefore both target genes of the same transcription factor but regulated in an opposite manner (Selvaraj *et al*, 2005). Whereas *Drosophila* Ctr1B is regulated at the transcriptional level, the human and yeast Ctr1 homologs appear to be post-translationally regulated. Copper excess stimulates rapid endocytosis and/or degradation of both yCtr1 and hCtr1 (Ooi *et al*, 1996; Petris *et al*, 2003; Guo *et al*, 2004), apparently to prevent over-accumulation and toxicity of copper. In a different study, hCtr1 remained stable and functional even after copper exposure (Eisses *et al*, 2005). Another transport system is able to maintain adequate activity under both low and high copper conditions. At normal or low copper, the related mammalian copper exporters ATP7A and ATP7B, also referred to as Menkes and Wilson P-type ATPases, respectively, transfer copper from the cytoplasm to the *trans*-Golgi network (TGN). At high copper, both exporters translocate from the Golgi to the cell surface, where they function to export copper from the cell (Petris *et al*, 1996). The importance of the latter process is illustrated by mutations in *ATP7B,* which causes Wilson's disease, where patients accumulate toxic amounts of copper in the liver (Bull *et al*, 1993; Yamaguchi *et al*, 1993). In spite of recent progress in our understanding of copper homeostasis, many questions remain concerning how copper levels are sensed and how they feed back to coordinate copper uptake, export or copper sequestration. In the fruit fly *Drosophila*, overall copper levels of the organism can vary at least by 20-fold depending upon the copper concentration in the diet (H Yepiskoposyan, A Simons and W Schaffner, unpublished). Apparently under high copper

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conditions, sequestration is a major mechanism ensuring a remarkable tolerance to copper. This tolerance depends on the copper sequestrating ability of metallothioneins and the transcription factor MTF-1, which activates transcription of the metallothionein loci from insects to mammals (Heuchel *et al*, 1994; Zhang *et al*, 2001). Accordingly, metallothionein or *MTF-1* mutants are highly sensitive to elevated copper concentrations (Egli *et al*, 2006a). In *Drosophila* larvae, metallothionein expression and the sites of copper accumulation coincide, especially in the cytoplasm of so-called copper cells of the midgut and also in the posterior midgut (Filshie *et al*, 1971; Tapp, 1975; Lauverjat *et al*, 1989; Marchal-Segault *et al*, 1990; Egli *et al*, 2006a). Noteworthy, copper cells display a characteristic orange copper luminescence caused by a copper–metallothionein complex (Egli *et al*, 2006a).

Here, we report that in spite of a downregulation of *Ctr1B* transcripts in high copper, the protein persists for many hours in a functional state. In *Drosophila* larvae, Ctr1B remains at the plasma membrane of intestinal cells and imports copper to high levels, causing a metal-stress response and the induction of metallothioneins. Taken at face value, this property indicates a flawed regulation of copper homeostasis. However, we provide evidence that transient copper accumulation in larvae can balance fluctuating copper availability in the food and helps to overcome intermittent periods of copper starvation, and is even used to supply copper to the following generation. Thus, the regulation of Ctr1B seems to reflect a trade-off between copper excess causing copper toxicity and the prospect of copper starvation, which severely delays development. We also show two additional levels of regulation, which help to limit copper accumulation: copper export via DmATP7, and a behavioral avoidance of food containing 0.5 mM or more copper by larvae.

Results

Metallothionein transcription as a sensitive indicator of heavy metal concentration and distribution

Drosophila larvae that are grown on copper-supplemented food contain high levels of metallothioneins, whereas metallothionein expression is low when larvae are grown on copper-depleted food (Figure 1A). Likewise, larvae that are constantly fed on zinc, cadmium or mercury containing media show a dose-dependent increase in metallothionein expression (Balamurugan *et al*, 2004). The tissue specificity of metal distribution coincides with metallothionein expression, for example, the so-called copper cells of the larval midgut accumulate high levels of copper and strongly express metallothionein genes. The endogenous metallothionein promoters or metallothionein-EYFP (enhanced yellow fluorescent protein) reporter constructs are thus sensitive indicators of metal transport processes within the intact organism.

Ctr1B is the most efficient copper importer in Drosophila larvae

The copper transporter Ctr1B is expressed during larval stages in the posterior midgut and mediates copper uptake from the food. We determined the induction of the metallothionein A (*MtnA*) promoter by copper and zinc in *Ctr1B* mutants and in wild-type larvae that were grown on normal food (NF) and transferred for 6 h to food supplemented with copper. Whereas wild-type larvae showed a strong induction

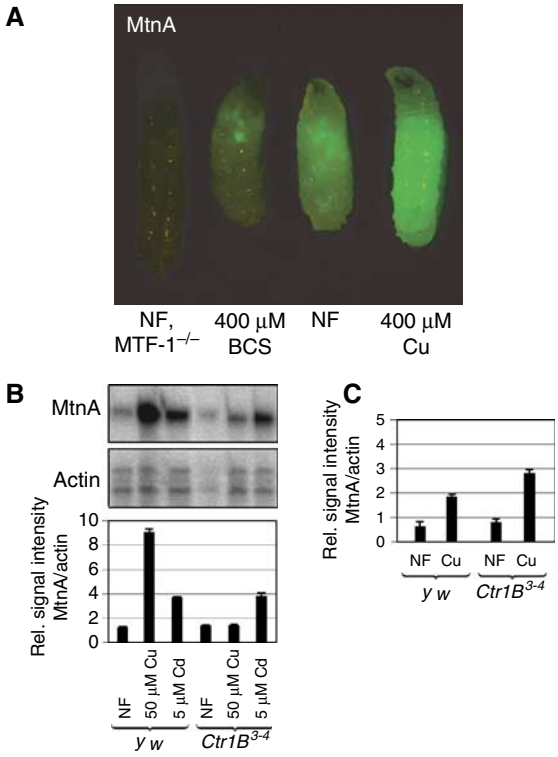

Figure 1 A metallothionein-EYFP reporter as a sensitive indicator of metal content and transport processes. (**A**) Expression of reporter transgene consisting of the *MtnA* promoter driving expression of EYFP. Shown are third instar *Drosophila* larvae grown on the indicated type of food. *MTF-1* mutant larvae, which lack metallothionein expression, owing to absence of the major transactivator MTF-1 (metal responsive transcription factor 1 or metal regulatory transcription factor 1), also do not show reporter gene expression as measured by fluorescence microscopy. (**B**) Mutants for the *Ctr1B* copper importer are deficient in copper-mediated induction of the *MtnA* promoter. Larvae at the third instar were transferred from normal to cadmium or copper containing food for 6 h and mRNA expression was determined by S1 nuclease mapping. Bars represent the quantification of *MtnA* mRNA levels normalized with the signal obtained with a probe complementary to *actin* mRNA. Results are the mean ± s.e.m. of three independent experiments. (**C**) Mutant and 'wild type' (*y w*) larvae that were continuously raised until third instar on normal food (NF) or copper containing food. Transcripts were quantified as in panel B.

of metallothionein transcripts by copper and cadmium, *Ctr1B* mutant larvae did not display copper-mediated induction, but maintained cadmium-mediated induction of metallothioneins (Figure 1B). This demonstrates that Ctr1B is the major intestinal copper importer of larvae growing on normal food. Interestingly, when larvae were constantly raised in high copper, metallothionein levels were as high in *Ctr1B* mutants as in wild type (Figure 1C). At first sight, these results seem to contradict each other. However, metallothionein induction in the first experiment relied on an efficient, rapid import of copper into the cell, whereas in the second, copper could accumulate over a period of several days. Our results suggest that under the latter conditions, copper import occurs independent of Ctr1B by less abundant, less efficient or less specific copper importers, possibly Ctr1A, Ctr1C or Malvolio (a *Drosophila* homolog of Nramp-1) (Rodrigues *et al*, 1995), ultimately leading to equal levels of Mtn induction in both *Ctr1B* mutant and wild-type larvae. Consistent

with this observation is the report that *Ctr1B* mutants contain less copper than wild-type flies under normal conditions, but the same amount under copper load (Zhou *et al*, 2003).

Rapid copper import by Ctr1B causes a metal-stress response

Ctr1B remained active when larvae were transferred from normal food to food containing high copper concentrations, which strongly induced metallothionein gene expression. Thus, we asked whether high expression of Ctr1B would cause a copper stress response even with normal food. Larvae that are grown in the presence of the copper chelator bathocuproine disulfonate (BCS) show high levels of Ctr1B to ensure sufficient copper uptake (Selvaraj *et al*, 2005). At the same time, transcription of metallothionein genes is low. Indeed, when copper-depleted larvae were transferred to normal food without copper chelator for 6 h, expression of metallothionein genes was strongly induced owing to high Ctr1B expression. This indicates that import of copper by Ctr1B leads to a copper-mediated stress response characterized by metallothionein gene induction (Figure 2A).

Metallothioneins reduce toxicity of a Ctr1B-mediated copper shock

As Ctr1B appears to import copper to critical levels, we wished to know whether transfer of larvae from low to high copper results in copper toxicity. To this end, we made use of a copper-sensitive 'quadruple metallothionein' (qMtn*) mutant strain that lacks all four metallothioneins (Egli *et al*, 2006a). These flies are viable and develop normally in up to 100 μM copper in the food, that is, they tolerate a certain amount of copper excess (although less than 5% of what wild-type larvae tolerate). We transferred copper-depleted or normally fed third instar larvae to food with high copper and scored for survivors that made it to adulthood. Only one-third of *qMtn* mutant larvae that were copper-depleted and therefore had high starting levels of Ctr1B survived the copper shock. *qMtn* mutant larvae that were grown in standard or copper-rich food before the transfer, and therefore had lower starting levels of Ctr1B, were more likely to survive (Figure 2B). As copper uptake via importers other than Ctr1B (Ctr1A and Ctr1C) is low under these experimental conditions (Zhou *et al*, 2003), we conclude that Ctr1B continues to import copper even after normal levels are reached, resulting in copper excess and, especially in the absence of metallothioneins, imminent toxicity. Wild-type control larvae survived a transfer from low to high copper quite well, indicating that metallothioneins play an important role in *buffering fluctuating* copper availability and uptake (Figure 1B; see also Egli *et al*, 2006b). We have also measured endogenous *Ctr1B* transcript levels in metallothionein mutants after transferring early third instar larvae from copper-depleted or normal food to high-copper food. We note that upon copper shock, *Ctr1B* transcript levels are similarly reduced in wild-type and metallothionein-knockout larvae, indicating that the copper importing activity of Ctr1B is not altered in the latter (Figure 2C). These observations corroborate our previous findings that the copper content of both wild-type and quadruple metallothionein-knockout flies is the same after copper shock (Egli *et al*, 2006a).

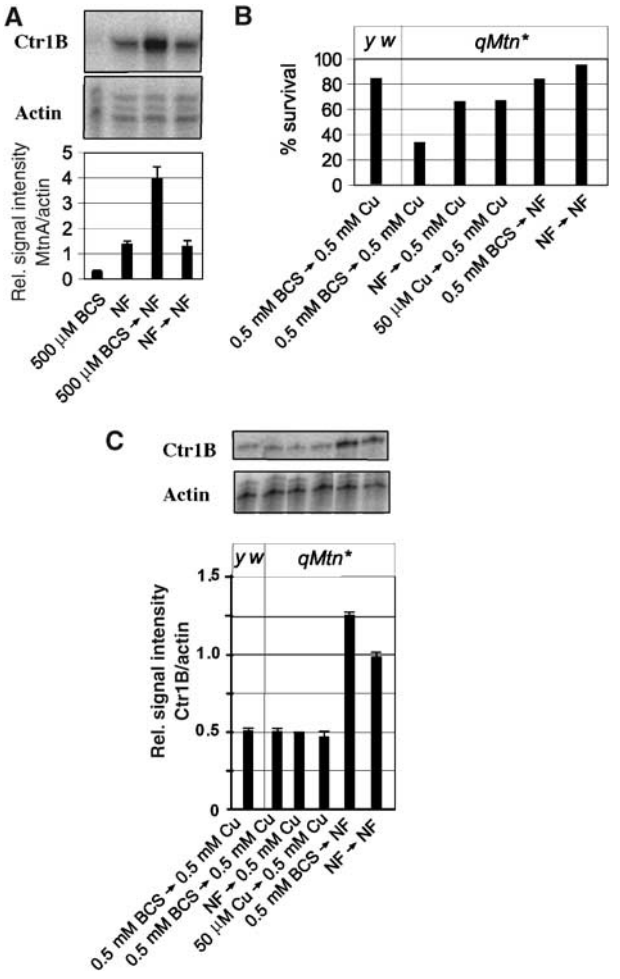

Figure 2 Copper shock: a cellular stress response owing to massive copper import. (**A**) *Drosophila* larvae were copper-starved by growth on food containing 0.5 mM copper chelator (BCS) or on normal food (NF) and transferred to NF for 6 h. Shown are the expression levels of metallothionein A (*MtnA*). Quantification was done as in Figure 1. (**B**) Wild-type (*y w*) or mutant flies with inactivation of all four metallothionein genes (*qMtn**) were grown for five days on 0.5 mM BCS and 30 third instar larvae were transferred to either normal food or 0.5 mM copper. Shown is the survival to adulthood in percentage. Results are the mean ± s.e.m. of three different experiments. (**C**) Wild-type or flies lacking all four metallothioneins (qMtn*) were first grown in the indicated type of food and allowed to lay eggs. Third instar larvae were transferred to high copper or normal food for 18 h. Total RNA was extracted and transcripts were quantified by S1 nuclease mapping. *Actin5c* RNA was used as a standard for normalization. Results are the mean ± s.e.m. of three independent experiments.

Ctr1B is mostly, if not exclusively, regulated at the transcriptional level

Copper chelation by metallothioneins reduces copper toxicity following a copper shock. In addition, we considered post-translational regulation of Ctr1B, for example, copper-dependent degradation, which was reported for mammalian Ctr1 (Petris *et al*, 2003). To find whether there was a similar regulation in flies, we transferred larvae from low (250 μM BCS) to high copper (250 μM Cu) and tested mRNA and protein levels of Ctr1B-EGFP. The transcriptional response was rapid: mRNA levels of *Ctr1B-EGFP* were reduced after merely 2 h, whereas at the same time, metallothionein A mRNA increased in parallel (Figure 3A). In contrast, Ctr1B-

EGFP protein levels remained constant even after 18 h of copper load (Figure 3B). In these experiments, we tested the levels of Ctr1B-EGFP fusion construct (AH3) where the EGFP coding region is fused in-frame to the last codon of a complete *Ctr1B* gene as well as endogenous Ctr1B (not shown). The Ctr1B-EGFP fusion construct was previously shown to be regulated by copper at the transcriptional level and to rescue all known aspects of the *Ctr1B* mutant phenotype, namely, sensitivity to both copper scarcity and to very high copper concentration (Selvaraj *et al*, 2005). Nevertheless, AH3 transgenic flies in a Ctr1B wild-type background were not more sensitive to copper shock than wild-type (*y w*) control larvae, indicating that the fusion construct does not result in a hyperactive allele and excessive copper import (not shown). Taken together, these findings suggest that *Drosophila* Ctr1B is not degraded in a copper-dependent manner; rather, the amount of Ctr1B is mostly, if not entirely, regulated at the transcriptional level.

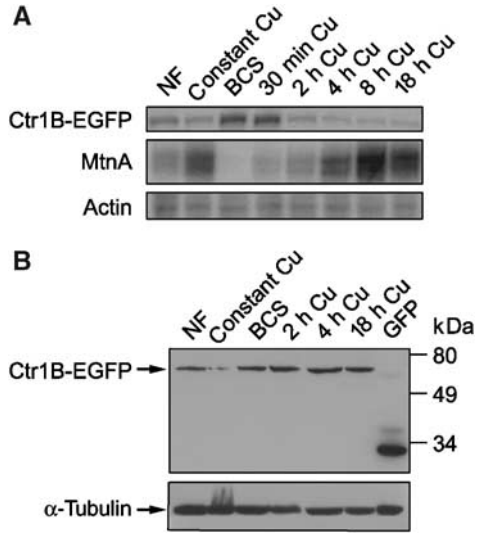

Figure 3 Ctr1B protein but not mRNA levels are stable after transfer of larvae from low to high copper. The different time points indicate the time after removing the larvae from low copper (i.e., BCS containing) food. The concentration of copper or BCS was 250 μM. (**A**) mRNA levels of MtnA or a Ctr1B reporter construct (Ctr1B-EGFP with the endogenous Ctr1B promoter) determined by S1 nuclease mapping. Note the opposite regulation of the two transcripts by copper availability (see also Selvaraj *et al*, 2005). (**B**) Western blot of Ctr1B-EGFP and α-tubulin. Unlike mRNA levels, Ctr1B protein levels are hardly affected by copper load during the first 18 h. NF = normal food.

The Ctr1B-EGFP fusion protein remains on the plasma membrane upon copper shock

Consistent with a role in copper uptake from the food, the Ctr1B-EGFP fusion protein localized to the apical side of the plasma membrane of larval gut cells (Figure 4A). To find out whether Ctr1B undergoes internalization at elevated copper levels as described for mammalian Ctr1 (Petris *et al*, 2003), we transferred third instar larvae with the Ctr1B-EGFP transgene from 100 μM BCS to 250 μM copper. Even after 24 h, when copper import has led to a strong activation of metallothionein genes, the Ctr1B-EGFP fusion protein persisted at the apical plasma membrane of intestinal cells (Figure 4B). Importantly, these cells also showed the characteristic copper luminescence, indicating that high amounts of intracellular copper were complexed by metallothioneins. Such high amounts of copper are not required for larval development, as larvae that are grown at mild copper depletion (100 μM BCS) also develop normally and maintain the same copper content as controls owing to the upregulation of Ctr1B (see below). These results show that copper does not alter the cellular localization of Ctr1B and provide cytological evidence that Ctr1B continues to import copper from the food beyond the immediate physiological requirements.

Copper excess is used to cope with periods of nutritional copper scarcity

To test whether stored copper helps to overcome a period of copper scarcity, we shifted larvae from copper-scarce to copper-supplemented food and then back to copper-scarce food. Growth on normal food until pupariation takes about 5 days, but growth on low copper can delay development by twice the normal time period or even more (Figure 5A). The developmental retardation is most pronounced in rapidly growing larvae. High copper concentrations can also delay

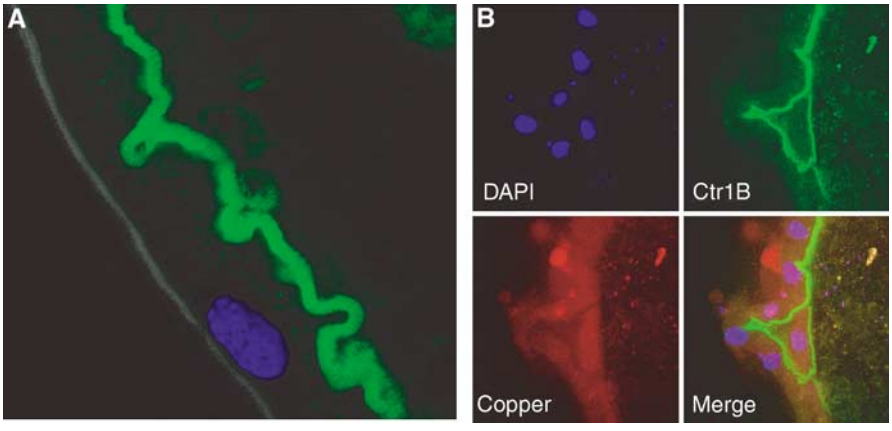

Figure 4 Apical localization of Ctr1B in intestinal cells is not regulated by copper. (**A**) Localization (apical) of the Ctr1B-EGFP fusion protein on the brush border membrane of an intestinal cell in the posterior midgut. The lumen of the gut is to the right. This larva was raised to the third instar on 100 μM BCS. Blue: DAPI staining of nucleus. (**B**) Copper shock does not alter the cellular localization of Ctr1B-EGFP. Third instar larvae were transferred from 100 μM BCS to 250 μM copper for 24 h and larval guts were analyzed by confocal microscopy. Copper accumulation is revealed by orange copper luminescence. The lumen of the gut, with some autofluorescent food particles, is to the right.

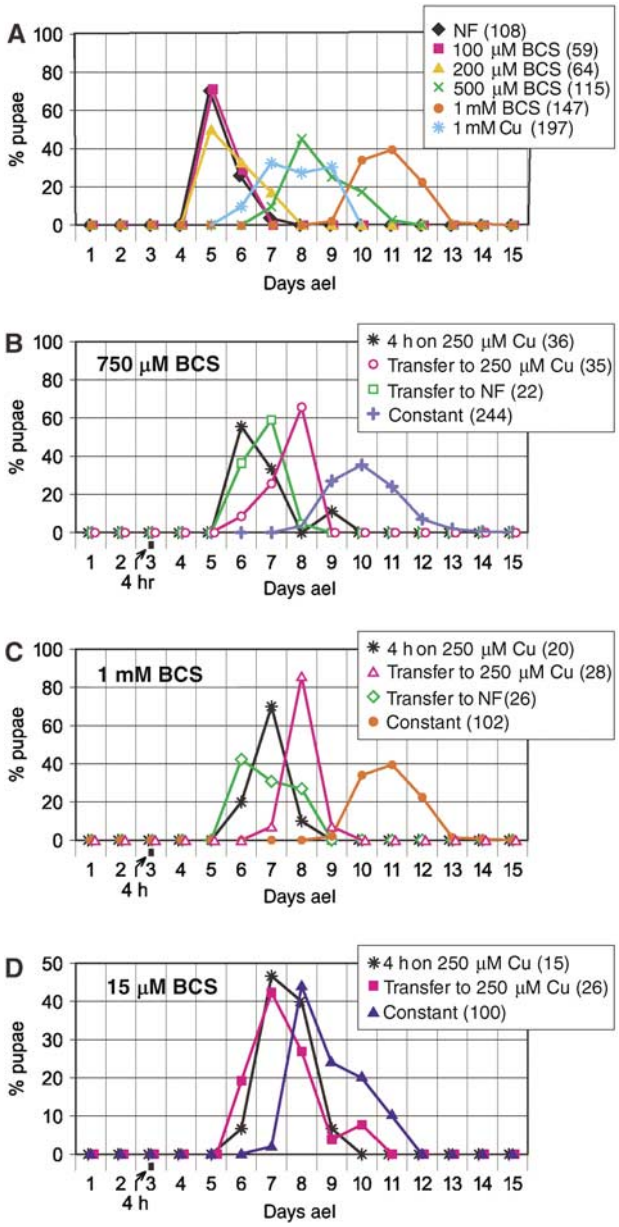

Figure 5 Copper starvation strongly delays development, but is rescued by a short copper pulse. Shown is the percentage of pupae forming on each day, counted after egg laying. The numbers in parentheses indicate the number of pupae in each experiment. (**A**) Wild-type flies were raised continuously on the indicated type of food. Note the severe delay in development by copper scarcity and also by copper load. (**B–D**) Larvae were transferred on the third day after egg laying (ael) to normal or copper-enriched food for further development, or kept in high-copper food for just four hours and then transferred back to the type of food indicated in each panel. (B, C) Wild-type $Ctr1B^{+/+}$ flies, (D) $Ctr1B^{-/-}$ flies.

growth (see also Egli *et al*, 2006a). Homeostasis of copper is therefore required to minimize the time span of complete development. To investigate the ability of *Drosophila* larvae to cope with nutritional copper fluctuations, we transferred 3-day-old second instar larvae from 750 μM BCS (wild type) or 15 μM BCS (*Ctr1B* mutants) to 250 μM Cu, or to normal food, either for 4 h or for the rest of the larval development, and measured the time needed to reach pupal stage (Figures 5B–D). The short 4 h copper pulse dramatically accelerated development of wild-type larvae by about 4 days compared to constant growth on 750 μM BCS. This result is comparable to growth of larvae shifted to normal food for the rest of development. A shift that was followed by constant growth on copper-enriched food however does not accelerate development (Figure 5B and C). Consistent with the role of Ctr1B as an intestinal copper importer, *Ctr1B* mutant larvae had great difficulties in exploiting such a transient period of copper abundance and were clearly retarded relative to equally treated wild-type larvae (Figure 5C). These results show that Ctr1B mediates efficient copper import within a short time window, ensuring a sufficient copper supply for the rest of development. This confers a substantial growth advantage when copper becomes scarce, but may also increase the risk of copper-mediated oxidative damage.

Accumulation of copper in the body: another key component in Drosophila copper homeostasis

Intestinal cells have a remarkable ability to induce transcription of metallothionein genes and to accumulate and detoxify high amounts of copper. In *Drosophila* larvae, Ctr1B is expressed in the intestinal cells of the posterior midgut, where metallothionein induction is high upon copper load (this study). To determine the effect of ectopic Ctr1B expression, we have tested a transgene where Ctr1B is under the control of an eye-specific *GMR* promoter. Such an ectopic (over)expression of Ctr1B resulted in distorted eye development, manifesting itself as 'rough eyes'. This phenotype critically depends on copper availability: copper scarcity alleviated the rough eye phenotype, whereas copper supplementation of the food aggravated it in a dose-dependent manner (Figure 6A). We also observed that the rough eye phenotype is more severe in flies that were grown in food containing both 20 μM copper and 0.02% hydrogen peroxide as compared to 20 μM copper or 0.02% hydrogen peroxide alone (Figure 6B), probably as a result of radical generation via the Fenton reaction (Halliwell and Gutteridge, 1990; Puig and Thiele, 2002).

To find out whether overexpression of MTF-1 and metallothioneins affected the rough eye phenotype, we coexpressed Ctr1B with transgenes encoding either metallothionein A or dMTF-1, using the eye-specific *GMR* promoter. Consistent with their role in copper detoxification, overexpression of metallothionein or dMTF-1 rescued the toxic effects of excessive copper import in the eye, whereas a dominant-negative version of dMTF-1 that maintains DNA binding but lacks activator function yielded a more severe phenotype (Figure 6A). Metallothioneins show their highest expression levels in the gut and are also expressed in the fat body upon copper load, but their expression is below detectable levels in imaginal discs and the brain. In line with this finding, imaginal discs do not accumulate metal under normal conditions (Ballan-Dufrancais, 2002), and may lack an efficient defense system. Therefore, copper distribution as well as the expression of proteins involved in copper homeostasis is tissue-specific. Some tissues, especially larval tissues that do not develop into the adult organism, tolerate high levels of copper and are apparently used to keep copper from the more sensitive tissues. Copper-accumulating tissues probably donate regulated amounts of the stored copper to copper-sensitive tissues during copper scarcity. Indeed, we observed that copper luminescence in the larval midgut that

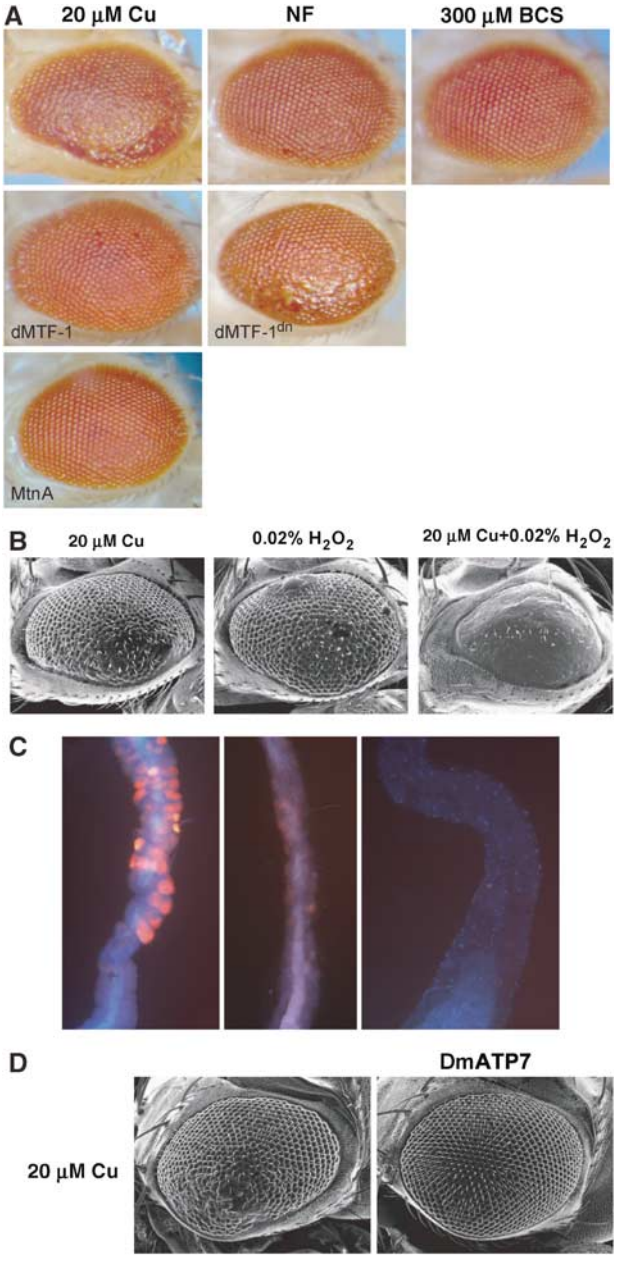

Figure 6 Ectopic copper import interferes with development. (**A**) Phenotype of flies with ectopic copper import into developing eye imaginal cells. Ctr1B is expressed under the control of the eye-specific GMR promoter in all panels. Shown are the scanning electron micrographs of the *Drosophila* eye. Flies were grown either in NF or 20 μM copper-supplemented food. 5 day-old flies were fixed in 2% osmium tetroxide for 1 h and then subjected to scanning electron microscopy. MTF-1, MTF-1^{dn} and MtnA designate fly lines with the co-overexpression of additional transgenes (dn = dominant negative form). (**B**) Synergistic effect of copper and hydrogen peroxide on rough eye phenotype. Flies were grown in food containing either 20 μM copper or 0.02% hydrogen peroxide (H_2O_2) or both. (**C**) Decreased copper luminescence in the intestine of larvae following transfer from food containing 250 μM copper to normal food. Shown is the confocal microscopic picture of the *Drosophila* larval midgut. (**D**) The phenotype caused by the expression of Ctr1B in the eye is rescued by co-expression of the *Drosophila* copper exporter DmATP7. Scanning electron micrographs of the eye of a Ctr1B overexpressing fly (left panel) is compared to the one of a Ctr1B and DmATP7 co-overexpresing fly (right panel).

marks sites of copper accumulation disappears within about 10 h upon withdrawal of copper from the food, showing that copper is not irreversibly trapped in copper-binding proteins in intestinal cells (Figure 6C). It remains to be seen what fraction of the copper is transported to other parts of the body and what, if any, is excreted, but it seems safe to state that copper storage is a dynamic process.

Role of copper transporter DmATP7 (Drosophila Menkes/Wilson's disease homolog) in counteracting copper toxicity

Besides the regulation of copper import, copper sequestration by metallothioneins and copper avoidance, copper export also plays a role to defend against copper toxicity. The *Drosophila* genome encodes a single homolog of the two related mammalian copper exporters ATP7A and ATP7B, also referred to as Menkes and Wilson's disease proteins, respectively (Southon *et al*, 2004; Norgate *et al*, 2006). We overexpressed the *Drosophila* homolog DmATP7 in the *Drosophila* eye by the UAS-Gal4 system. Overexpression of DmATP7 does not cause an eye phenotype *per se* but alleviates the toxicity of excessive copper import mediated by Ctr1B (Figure 6D). Detoxification most likely occurs via removal of copper from affected cells, indicating that copper export is yet another defense mechanism against copper excess, similar to the excretion of copper in the liver by the related mammalian Wilson's copper transporter ATP7B. While these experiments were underway, a detailed study on the role of DmATP7 in *Drosophila* copper homeostasis came to the same conclusions (Norgate *et al*, 2006).

Copper can be transferred to the next generation

In spite of the mechanisms used by *Drosophila* to alleviate copper toxicity, in balance it seems best to maximally fill the copper stores, because even the following generation can profit from the supply. To test this hypothesis, *Drosophila* larvae were raised in copper-supplemented food and the resulting flies were transferred to copper-depleted food for egg deposition. Such offspring grew faster (not shown) and survived better than the offspring of control flies (Figure 7). Strikingly, this beneficial effect extended to a further generation: offspring of second-generation low-copper flies raised again in low copper fared much better if their grandparents had been grown up in copper-supplemented food (Figure 7, lanes G3).

Drosophila larvae avoid potentially toxic levels of copper

The ability of Ctr1B to excessively import copper suggests the presence of other mechanisms to protect the organism from copper toxicity. We therefore tested the behavioral response of wild-type larvae to copper. Third instar larvae raised in normal food were placed in a Petri dish and given the choice between two types of semi-solid food (Figure 8A). One-half of the dish contained normal food, whereas food on the other half was supplemented with different copper concentrations or other transition metals. Irrespective of whether larvae were initially placed on the half containing normal food or copper-enriched food, they rapidly redistributed over the whole plate in an initial phase. However, after prolonged crawling, they obviously sensed and avoided the copper-containing half, such that only 10–20% localized to that

half after 60 min. While a copper concentration of 0.25 mM copper elicits at most a mild avoidance, a clear avoidance was observed at 0.5 or 1 mM copper. Different anions, either $CuCl_2$ or $CuSO_4$, yielded very similar results (not shown).

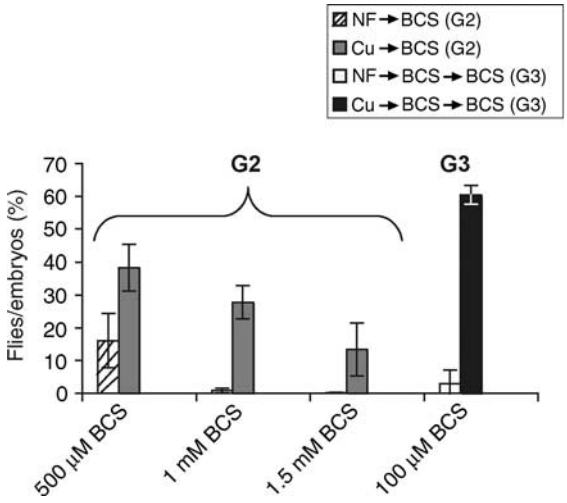

Figure 7 Copper supply to following generation. *Drosophila* larvae of generation 2 (G_2) thrive on copper-starved food if their parents had been raised during larval stage in copper-supplemented food. Controls whose parents grew up in standard food are severely delayed (not shown), and many fail to develop to adulthood, as illustrated by the ratio of flies that eclosed from originally laid eggs on various concentrations of copper chelator (BCS). The beneficial effect of copper transfer to G2 even extends to a further generation (G3) raised again in low-copper food.

Drosophila larvae also avoided food with 1 mM silver, but were indifferent to 4 mM zinc, 0.5 mM cadmium or 5 mM iron (Fe^{3+}) (Figure 8B and C). These controls demonstrate that the avoidance reaction is specific to copper, or in general to elements of group Ib (which includes silver) in the periodic table of elements and not due to a difference of osmolarity between the metal-containing half and the normal half. None of the metal concentrations tested affected viability during the 2 h interval of the food-choice test; 4 mM zinc, 0.5 mM copper or 5 mM iron was well tolerated by wild-type larvae even upon constant exposure to these metals, whereas 0.5 mM cadmium or silver was toxic to larvae upon prolonged exposure (not shown). We also tested whether the avoidance of copper and silver depends on the function of either the regulator MTF-1 or the copper importer Ctr1B. However, both *MTF-1* and *Ctr1B* mutant larvae displayed the same copper avoidance as wild type, suggesting that another copper importer or some kind of a copper receptor on the cell surface triggers this behavioral response (data not shown).

Discussion

Our results imply that copper levels in the food or in the cell do not alter the localization of the Ctr1B copper importer protein in *Drosophila*. Instead, copper import by Ctr1B is mostly, if not exclusively, regulated at the transcriptional level by copper availability in an MTF-1-dependent manner. This form of regulation is in apparent contrast to yeast Ctr1, which is regulated post-translationally (Ooi *et al*, 1996). Concerning human Ctr1, one study has shown that it under-

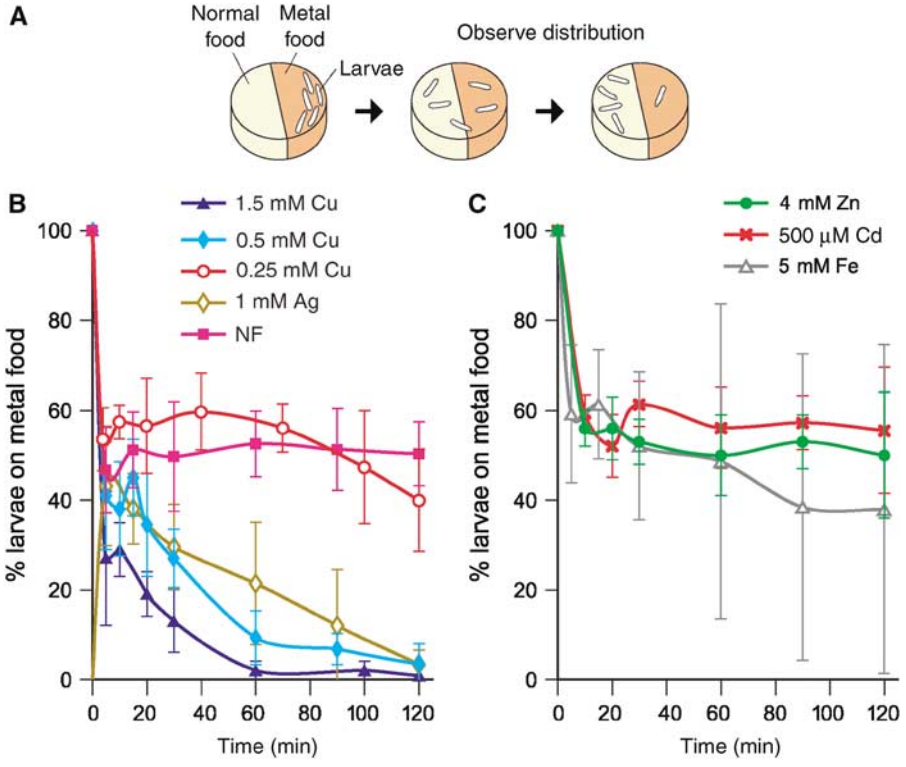

Figure 8 Behavioral avoidance of copper. (**A**) Groups of 30 larvae were placed on the metal-containing half food in a Petri dish, from where they crawled on the surface of solid food in search of a hole to enter the 'food cake', thereby given the choice between metal containing and normal food. At the time points indicated, larvae were counted in each half. The colors of the two halves differ in this Figure for didactical purposes only. (**B, C**) Shown is the percentage of larvae crawling on the metal-containing food as a function of time.

goes degradation in response to copper (Petris *et al*, 2003), whereas another reports that it stably resides on the plasma membrane (Eisses *et al*, 2005). Interestingly, some other Ctr family members, including hCtr1 and yCtr1, possess 'Mets motifs' (MxxM or MxM) that are not present in *Drosophila* Ctr1B and yCtr3, or in paCtr3 of the filamentous fungus *Podospora anserina* (Ooi *et al*, 1996; Koch *et al*, 1997; Pena *et al*, 2000; Guo *et al*, 2004). Several studies have shown that these 'Mets motifs' are pivotal for copper-dependent endocytosis (Guo *et al*, 2004; Jiang *et al*, 2005). Consistent with the idea that the 'Mets motifs' are necessary for copper-stimulated endocytosis and degradation, yCtr3, in contrast to yCtr1, does not undergo copper-dependent endocytosis (Pena *et al*, 2000). Indeed yCtr3, Ctr1B and paCtr3 are all transcriptionally activated under conditions of copper scarcity by their respective transcription factors Mac1 (which also activates yCtr1), MTF-1 and Grisea (Graden and Winge, 1997; Labbe *et al*, 1997; Borghouts *et al*, 2002; Selvaraj *et al*, 2005). The two other *Drosophila* copper importers Ctr1A and Ctr1C, whose role in copper homeostasis remains to be elucidated, are not regulated at the transcriptional level but contain Mets-like motifs and may thus be regulated at the protein level by copper (Zhou *et al*, 2003). In the present study, we show that copper does not trigger endocytosis of *Drosophila* Ctr1B, and that Ctr1B continues to import copper irrespective of the ambient copper concentration. In agreement with such a differential mode of regulation, Ctr1B is the closest *Drosophila* homolog of yCtr3, whereas Ctr1A and Ctr1C are more closely related to yCtr1 (Ooi *et al*, 1996; Koch *et al*, 1997; Pena *et al*, 2000; Zhou *et al*, 2003).

Drosophila has a remarkable ability to accumulate copper. This accumulation is mediated by the major larval copper importer Ctr1B upon an increase of copper levels in the diet, as Ctr1B remains on the brush border membrane of gut cells. Copper accumulation strongly induces transcription of metallothioneins, with subsequent copper sequestration and storage primarily in intestinal cells. The stored copper can serve as a source in periods of copper scarcity, whether in the same or, surprisingly, even in the following generation(s) (Figure 7). Intestinal accumulation of copper also helps to protect other tissues such as brain and imaginal discs, whose development or function might be disrupted by copper load (Rodrigues *et al*, 1995; Folwell *et al*, 2006; Egli *et al*, 2006a). Indeed an increased copper import by ectopic expression of Ctr1B in the eye interferes with its development, resulting in a characteristic 'rough eye' phenotype.

An important question is whether the seemingly sluggish regulation of Ctr1B that can result in a copper-stress response serves a purpose under natural conditions or whether a more precise regulation is simply not necessary. A sufficient uptake and the ability to store copper may be particularly important in geographical regions where copper is scarce. Copper scarcity results in a strong delay of larval development, a condition that may be of selective disadvantage in a rapidly changing environment. Fluctuations of copper content in the nutrients may be quite common, at least locally: *Drosophila* had been exposed to high copper levels over the course of the last century as copper-containing sprays are used in vineyards and orchards to combat bacterial and fungal infections (Maroni *et al*, 1987). A downregulation of Ctr1B upon a change from untreated to copper-treated fruits appears necessary but copper shock can also be controlled by the copper avoidance reaction. Therefore, *Drosophila* may get by with a sluggish downregulation under most circumstances.

Copper, like other essential trace elements or nutrients such as amino acids or lipids, is a limiting factor for development even when all other nutrients are present in abundance. To avoid such a situation, most organisms have the ability to store nutrients, for example, lipids in fat tissue, sugars as glycogen and iron in ferritins, iron-binding proteins (Harrison and Arosio, 1996). Similarly, metallothioneins can be considered as stores of essential transition metals, notably copper. Ctr1B would simply fill these stores as long as copper is abundant. Because of this potential benefit, we propose that *Drosophila* allows copper accumulation, which is accompanied by a copper-mediated physiological stress response. The accumulated copper, in spite of its tight binding to metallothioneins, could be made available in periods of copper scarcity by oxidation of metallothioneins in a manner akin to that described for copper-thioneins in mammals (Feldman and Cousins, 1976; Bremner *et al*, 1978; Liu *et al*, 2000). The released copper may be distributed to other parts of the body via DmATP7 analogous to mammalian ATP7A, which exports copper from intestinal cells through the basolateral membrane to the bloodstream (Petris *et al*, 2003; Southon *et al*, 2004; Norgate *et al*, 2006). DmATP7, like mammalian ATP7A, is required for proper pigment formation by the copper-containing enzyme tyrosinase (Petris *et al*, 2003; Norgate *et al*, 2006). The importance of copper accumulation and distribution is illustrated by the amazing ability of *Drosophila* to thrive on low-copper food if the parents had been raised during their larval development in copper-supplemented food. We propose that copper accumulation and transfer to the next generation ensures a sufficient copper supply and is of primary importance for the organism, whereas the threat of copper toxicity may be secondary. It remains to be determined which component(s) of the copper homeostasis system is responsible for this trans-generation effect.

The behavioral aspect of copper homeostasis is also intriguing. In *Drosophila*, copper avoidance, which was also observed in other organisms (Rabin *et al*, 1985; Sambongi *et al*, 1999; Lopes *et al*, 2004; Van Zwieten *et al*, 2004), occurs at relatively high levels of copper that would lead to a copper-mediated developmental delay of wild-type flies. The threshold of about 0.5 mM copper to trigger the avoidance probably reflects a tradeoff between accumulation to overcome periods of copper scarcity and the prospect of copper-mediated damage. Taken together, regulated copper import with specific chelation and storage, export, and also copper avoidance, establish an adequate homeostasis of this essential but potentially toxic trace metal.

Materials and methods

Fly stocks and genetics

The *dMTF-1* (*dMTF-1*$^{140-1R}$) and *Ctr1B* (*Ctr1B*$^{3-4}$) mutant alleles, the Ctr1B reporter transgenes and the *UAS-Ctr1B* transgene used for ectopic expression of Ctr1B were described in previous studies (Egli *et al*, 2003; Zhou *et al*, 2003; Selvaraj *et al*, 2005). As all experiments were carried out in a *y w* background that is often used for *Drosophila* experiments, the term 'wild type' is meant to indicate wild-type status of the relevant genes of copper homeostasis. We used Oregon R *y w* strain in all our experiments. The full-length as well as the dominant-negative MTF-1 lacking the transcriptional activation domains C-terminal to the zinc fingers

was cloned into the widely used pUAST vector. The EP insertion into the *DmATP* locus was kindly provided by Marcel Zarske and Ernst Hafen. Overexpression of these transgene in the eye was performed by crossing to a GMR-Gal4 transgenic fly. Experimental procedures of copper shock and transfer experiments are indicated in the figure legends.

MtnA-EYFP reporter construct

The *MtnA* promoter (−446 to +74) was PCR amplified from genomic DNA using the primer pair 5′-CGG GAT CCA GGT ATG GGC TAT TTA GGC C-3′ and 5′-GGG ATG GCC CCA AAG GAT CTG-3′. The PCR product was cloned in pCasper4 vector containing the coding region of EYFP (Thummel and Pirrotta, 1992).

Quantification of metallothionein and Ctr1B transcripts, and metal measurements

Larvae were either continuously raised on the indicated type of food or transferred for 6 h to normal food or metal-supplemented food (for simplicity, the terms or symbols copper, cadmium, silver, zinc and iron are meant to refer to Cu^{2+}, Cd^{2+}, Ag^{+}, Zn^{2+} and Fe^{3+}, respectively). Only third instar feeding larvae were used for analysis. Total RNA was extracted using the TRIzol reagent (Life Technologies). S1 nuclease mapping of transcripts with 50 μg of total RNA was performed as described previously (Weaver and Weissmann, 1979). The gels were developed using PhosphorImager (Molecular Dynamics) and bands were quantified. The signal from the endogenous *actin5c* gene was used for normalization of metallothionein transcript levels. Metal measurements were made as described elsewhere (Egli *et al*, 2006b).

Drosophila protein extracts and Western blot analysis

Transgenic flies harboring the full-length Ctr1B ORF fused to the EGFP-coding sequence (designated as AH3; Selvaraj *et al*, 2005) were allowed to grow on NF or food supplemented with 250 μM BCS or 250 μM copper. About 30–50 third instar larvae were taken for homogenization in a buffer containing 20 mM Hepes pH 7.5, 100 mM KCl, 5% glycerol, 100 mM EDTA, 0.1% Triton X-100, aprotinin (5 μg/ml), leupeptin (5 μg/ml), 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 2.5 mM dithiothreitol (DTT). The mixture was spun, the supernatant was collected, 50 μg of the extracts was resolved by 11% SDS–PAGE and Western blotting was performed according to standard procedures. The mouse anti-GFP was used at 1:2500 dilution (BD Biosciences, USA). For the GFP control, *Drosophila* S2 cells were transfected with pAc-GFP expression vector and whole-cell extracts were prepared 72 h after transfection. Blots were visualized using the ECL kit (Amersham Biosciences, Sweden).

Imaging and microscopy

Images were taken with a Leica MZ FLIII fluorescence stereomicroscope and a Nikon COOLPIX950 digital camera for whole larvae. Eyes were photographed using a Leica MZ16 stereomicroscope equipped with a Leica DFC280 camera and scanning electron microscopy images were taken with a JEOL JSM-6360LV scanning electron microscope. Copper cell luminescence and fluorescent protein expression in dissected larval guts were analyzed with a Leica DMRB fluorescence stereomicroscope equipped with the filters A for DAPI and copper cell luminescence and I3 for EGFP and EYFP, respectively. Pictures were taken with a Zeiss Axiocam. Confocal images were taken with a Leica SP1 UV CLSM.

Food choice and copper donation experiments

About 25–40 third instar larvae were placed on 5 cm Petri dishes. These contained two halves consisting of normal food or metal-supplemented food ($CuSO_4$, $CuCl_2$, $AgNO_3$, $ZnCl_2$, $FeCl_3$, $CdSO_4$). One liter of normal food consisted of 15 g agar, 16.6 g sugar (D-glucose), 10 g wheat, 25 g yeast and 330 ml fruit juice (90% apple juice and 10% pear juice). The agar concentration was chosen relatively high to prevent the rapid disappearance of larvae in the substrate.

For copper transfer studies, in each plastic vial, 20–30 wild-type adult flies were placed on either normal food or food containing 1 mM $CuSO_4$ and allowed to lay eggs. The resulting progeny that grew up in normal food or copper-supplemented food was designated as $G1_{NF}$ and $G1_{Cu}$, respectively. A total of 20–30 adult flies of each $G1_{NF}$ and $G1_{Cu}$ were placed on food containing copper chelator BCS for egg deposition, and were removed from the vials after 12 h. The number of eggs in each vial was determined and 10 days later the eclosed flies in that vial were counted. Similarly, G2 flies raised in BCS were transferred to a new vial with food, allowed to lay eggs and removed after 12 h. Again, the ratio of flies (G3) eclosing per originally deposited eggs was determined.

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Copper sensing function of *Drosophila* metal-responsive transcription factor-1 is mediated by a tetranuclear Cu(I) cluster

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ABSTRACT

Drosophila melanogaster **MTF-1 (dMTF-1) is a copper-responsive transcriptional activator that mediates resistance to Cu, as well as Zn and Cd. Here, we characterize a novel cysteine-rich domain which is crucial for sensing excess intracellular copper by dMTF-1. Transgenic flies expressing mutant dMTF-1 containing alanine substitutions of two, four or six cysteine residues within the sequence ^{547}CNCTNCKCDQTKSCHGGDC^{565} are significantly or completely impaired in their ability to protect flies from copper toxicity and fail to up-regulate MtnA (metallothionein) expression in response to excess Cu. In contrast, these flies exhibit wild-type survival in response to copper deprivation thus revealing that the cysteine cluster domain is required only for sensing Cu load by dMTF-1. Parallel studies show that the isolated cysteine cluster domain is required to protect a copper-sensitive *S. cerevisiae ace1*Δ strain from copper toxicity. Cu(I) ligation by a Cys-rich domain peptide fragment drives the cooperative assembly of a polydentate [Cu_4-S_6] cage structure, characterized by a core of trigonally S_3 coordinated Cu(I) ions bound by bridging thiolate ligands. While reminiscent of Cu_4-L_6 (L = ligand) tetranuclear clusters in copper regulatory transcription factors of yeast, the absence of significant sequence homology is consistent with convergent evolution of a sensing strategy particularly well suited for Cu(I).**

INTRODUCTION

Metal ions play myriad essential roles in all of biology. As a result, all cell types have evolved the ability to extract specific metal ions from their environment and ultimately maintain the intracellular concentrations of each in a range compatible with cellular needs (1). This is critical for the survival of the organism since even essential transition metal ions, e.g. Fe, Cu and Zn are toxic in excess (2). The same is true for Ni (3) and Mn (4), although acquisition of these ions ensures that specialized microorganisms are capable of surviving in a strongly acidic or potently oxidizing environment, respectively. Cu and Fe are particularly toxic since their reduced forms, Cu(I) and Fe(II), when weakly chelated in an aerobic environment, will catalyze the production of damaging hydroxyl radicals via redox cycling; as a result, the 'free' or bioavailable concentrations of these ions, as well as Zn, may likely be vanishingly small (5,6). The control of metal homeostasis is mediated by the balancing of uptake, efflux and intracellular sequestration or compartmentalization of essential metal ions, and is largely regulated transcriptionally by gene regulatory proteins, collectively coined metal sensor proteins (2). Metal sensor proteins directly bind a particular metal ion, or groups of metal ions that form similar coordination complexes, to the exclusion of

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all others (7); this, in turn, allows an organism to turn on or turn off the expression of specific genes in order to mount a metabolic response to either deprivation or excess of a particular metal ion in the cell.

Metal-responsive transcription factor-1 (MTF-1) is a heavy metal sensing transcriptional activator that up-regulates the expression of genes that allow an organism to mitigate zinc, cadmium and copper toxicity (8) (for reviews, see (9–13)). MTF-1 has been identified and at least partially characterized from human, mouse (14), pufferfish *Fugu rubripes* (15), zebrafish *Danio rerio* (16,17) and *Drosophila* (18). Human and mouse MTF-1 as well as the *Drosophila* homolog, termed dMTF-1, contain multiple functional domains, including a highly conserved zinc-finger domain that recognizes the cognate DNA sequence termed metal response element (MRE) (8). MTF-1 also harbors multiple domains for transcriptional activation (19), and short sequences that mediate intracellular trafficking into and out of the nucleus (20).

How a particular metal ion mediates MTF-1-dependent metalloregulation of gene expression is the subject of current debate (13,21); however, multiple levels of regulation clearly exist (20,22,23). Zn(II) binding to the zinc-finger domains clearly stabilizes an MRE-MTF-1 complex (8), particularly in chromatin (24). Biochemical studies of the finger domain fragment (25–27) have revealed that at least part of the zinc-sensing mechanism is mediated by the zinc-finger domain itself (21). However, MTF-1 also senses other cell stress conditions including Cd(II) (28), oxidative stress (29), hypoxia (30), and the synergistic influence of heavy metal load and heat shock (31). It seems unlikely that such inducers would act directly on the finger domain. For example, it is known that Cd(II) does not bind to the finger domain in a way that preserves the canonical ββα-structure for DNA binding (26). However, substantial data support an indirect sensing model, in which MTF-1 senses Zn(II) that is mobilized by other inducers from intracellular stores of cytoplasmic Zn(II) (29).

Previous functional studies of mammalian MTF-1 reveal that a 13-amino acid domain containing four conserved cysteines just C-terminal to a transcriptional activation domain is required for Zn(II)/Cd(II)-induced transcriptional activation in transiently transfected mouse MTF-$1^{-/-}$ cells (23). The mechanistic role of this domain in metalloregulation is not yet clear. However, it functions downstream of nuclear translocation and MRE-binding, perhaps activating transcription via a metal-dependent protein-protein interaction at the promoter. Indeed, when *Drosophila* S2 cells are stimulated with exogenous copper salts, dMTF-1 recruits TFIID to the *MtnA* (metallothionein A) promoter (32).

Drosophila MTF-1 differs from mammalian MTF-1 in two crucial respects. First, MRE- and MTF-1-dependent expression of metallothionein genes (*mtnA-D*) is strongly induced by Cu and Cd, relative to Zn, whereas Zn and Cd are the most potent inducers of mammalian metallothioneins (18). Second, disruption of the *MTF-1* gene by targeted insertional mutagenesis (MTF-1 KO flies) results in a strong sensitivity to not only Cu, Cd and Zn toxicity but also to Cu depletion (33,34). The requirement for dMTF-1 to mitigate the effects of Cu deprivation is unique to dMTF-1, and originates with the ability of dMTF-1 to activate expression of a high affinity Cu importer *Ctr1B* under normal or low-Cu growth conditions. As a result, dMTF-1 plays a central role in copper homeostasis in the fly by regulating both import and sequestration of this essential yet toxic metal (34,35).

We reasoned that some aspect of copper-dependent metalloregulation of dMTF-1 requires the direct binding of Cu(I), analogous to the direct binding of Zn(II) to the zinc fingers of hMTF-1. Such a Cu(I)-sensing mechanism is however unlikely to function through the zinc finger domain itself, which is predicted to have a low affinity for Cu(I); thus, some other Cu(I)-binding domain would have to be present in dMTF-1. Inspection of the amino acid sequence reveals two candidate cysteine-rich Cu(I)-binding domains, both located in the C-terminal one-third of the protein (9). Here, we present evidence that the six cysteine residues from residues 547-565 are necessary for dMTF-1 to sense copper load. When challenged with copper stress, flies harboring Cys-to-Ala substitutions are unable to up-regulate the transcription of metallothionein *MtnA*, the major effector of copper-resistance (36,37). We also show that a peptide harboring this Cys-rich domain protects a Cu-sensitive *S. cerevisiae* strain (38) from Cu-toxicity, presumably by mediating intracellular storage/chelation of the metal. Binding studies show that the Cu-sensing domain of dMTF-1 binds four Cu(I) ions tightly and highly cooperatively to form a Cu_4-Cys_6 polynuclear cluster. This cluster is reminiscent of known Cu-sensing domains of *S. cerevisiae* Mac1 and Ace1 and paralogs in other organisms (39–41). The mechanistic implications of these findings are discussed.

MATERIALS AND METHODS

Plasmids and fly transformation

Cys-to-Ala mutations were generated using pUAST-dMTF-1 as a template by a quick change mutagenesis technique. pUAST-dMTF-$1^{4C\text{-}4A}$, pUAST-dMTF-$1^{2C\text{-}2A}$ and pUAST-dMTF-$1^{6C\text{-}6A}$ constructs were used to generate transgenic flies with P-element mediated transformation as described earlier (37).

Fly food, fly stocks and genetics

One liter of standard fly food was composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent nipagin (15% in ethanol). For toxicity experiments, food was supplemented with $CuSO_4$ or CdCl2 or bathocuproinedisulfonate (BCS) disodium salt hydrate (Sigma-Aldrich No. 14,662-5) to the indicated concentrations. BCS is a specific copper chelator used to deplete copper in the food. Flies were raised at 25°C and 65% humidity. *UAS-dMTF-1*, *UAS-dMTF-*1^{4CA}, *UAS-dMTF-*1^{2CA}, and *UAS-dMTF-*1^{6CA} transgenes were crossed into *dMTF-*$1^{140\text{-}1R}$ (*dMTF-1* null allele) background respectively. The expression of the transgenes was induced by a ubiquitous Gal4 transactivator (*actin-Gal4*).

Drosophila toxicity experiments

The flies that were homozygous for *dMTF-1*$^{140\text{-}1R}$ and *UAS-dMTF-1* (or its derivatives) were crossed with *y w;; dMTF-1*$^{140\text{-}1R}$, *actin-Gal4/TM6B,y+* flies on standard food or food containing copper or BCS. From the cross, two types of progeny could be obtained: Progeny (A) flies that were expressing the transgene and were *dMTF-1* null mutant. Progeny (B) flies that were not expressing the transgene and contained endogenous *dMTF-1*. The survival index (*I*s) was calculated as follows: $Is = 2A/(A + B)$.

Quantitation of MtnA and dMTF-1 transcripts in transgenic flies

To determine the level of MtnA transcripts, larvae were raised on either standard food or food containing 100 μM $CuSO_4$. Only third instar stage larvae were collected for analysis. Total RNA was extracted using the TRIzol reagent (Life Technologies) and nuclease S1 mapping of transcripts (100 μg of total RNA) was performed as described previously (42). The gels were developed using FLA-7000 system and bands were quantified using ImageGauge software (Fuji Film). The transcripts of the endogenous *actin5c* gene were measured and used for normalization of MtnA transcript levels. To monitor *dMTF-1* expression levels, the gut tissue was dissected from the third instar stage larvae raised on standard food. Total RNA was extracted using TRizol and first-strand cDNA synthesis was performed with 5 μg total RNA using reverse transcriptase (RT). mRNA levels were measured by quantitative (q) PCR using a SybrGreen Q-PCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, *actin* mRNA was used as a reference.

Cu(I) binding experiments by absorption and luminescence spectroscopies

All Cu(I) titration samples of C-dMTF_81 were prepared anaerobically in a glovebox ($[O_2] < 2$ ppm) with deoxygenated buffers and solvents in 10 mM MES, 0.1 M NaCl, pH 6.3, 25°C. The samples were kept in sealed containers, including during transfer from the glovebox for characterization. Then, 500 μM Cu(I) was titrated into 800 μL of 20 μM apo-protein in anaerobic environment and the absorption was monitored over the wavelength range 200–500 nm on a Hewlett-Packard model 8452A spectrophotometer. In magfura-2 competition experiments, Zn(II) was titrated into the mixture of 15.8 μM C-dMTF_131 and 16.3 μM magfura-2 (43). For the competition experiments with BCS, 282 μM C-dMTF_81 was titrated into a mixture of 100 μM BCS and 30 μM Cu(I) and the absorption spectra recorded from 250 to 600 nm. Luminescence spectra were recorded on an ISS PC1 Photon Counting spectrofluorometer. Also, 1.0 mM Cu(I) was titrated into 1700 μL of 20 μM apo-C-dMTF_81 and the full emission spectra were collected from 400 to 800 nm with excitation at 300 nm essentially as described (44).

RESULTS

Domain structure of Drosophila MTF-1

The domain structure of *D. melanogaster* MTF-1 (dMTF-1) is shown in Figure 1 (18). The functional domains of dMTF-1 have not yet been extensively mapped and the amino acid sequence has diverged considerably from mammalian MTF-1 outside of the DNA-binding zinc finger domain (18). However, dMTF-1 contains a cluster of six cysteines within 19 consecutive amino acids (residues 547-565) that bears some

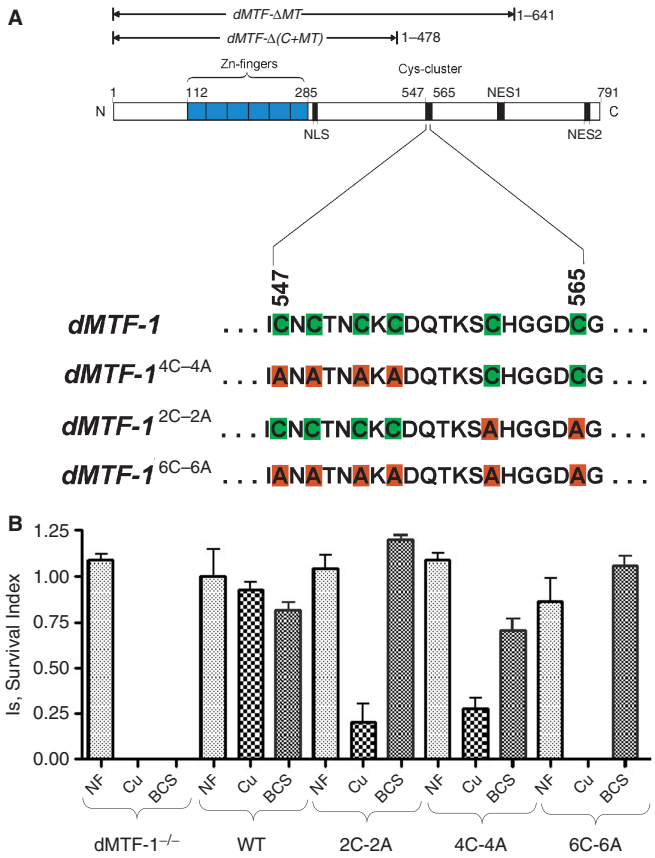

Figure 1. The cysteine-rich region plays a critical role in protecting *Drosophila* from copper toxicity. (**A**) Domain structure of *Drosophila melanogaster* (dMTF-1) highlighting the short Cys-cluster region (residues 547-565) and *dMTF-1* Cys-to-Ala substitution alleles characterized in transgenic flies. In addition to the zinc-finger DNA-binding domain, a putative nuclear localization signal (NLS) and two nuclear export signals (NES1 and NES2) are also indicated (V. Günther and W.S., unpublished). 131- (C-dMTF_131), 81- (C-dMTF_81) and 51- (C-dMTF_51) residue constructs of dMTF-1 characterized here correspond to amino acid residues 499-629, 499-579 and 529-579, respectively. Two C-terminal domain deletion mutants of dMTF-1, *ΔMT* and *Δ(C + MT)* characterized in *S. cerevisiae* (see Figure 3) are also shown. *MT*, metallothionein-like segment (residues 642-791); *C*, Cys-rich domain (residues 479-641). (**B**) Survival of *dMTF-1* null flies and flies expressing *dMTF-1*$^{2C\text{-}2A}$, *dMTF-1*$^{4C\text{-}4A}$ or *dMTF-1*$^{6C\text{-}6A}$ on a standard food source (NF), or on food supplemented with 400 μM $CuSO_4$ (Cu) or 160 μM bathocuprione disulfonate (BCS).

resemblance to the Cys_4 cluster that has been functionally characterized in hMTF-1 (23). This cluster is followed by a Thr/Ser-rich domain (13 Thr/Ser in 19 residues), which is connected via a seryl-glycyl linker to a C-terminal metallothionein (MT)-like domain, which also contains several cysteines (residues 641-791). This C-terminal MT-domain bears strong resemblance to domain IV of *S. pombe* Pccs, a copper chaperone for copper-zinc superoxide dismutase (SOD1) which have been shown to protect *S. pombe* and a *S. cerevisiae ace1*Δ mutant strain from copper toxicity (45). To probe the copper-binding ability of the cysteine cluster (residues 547-565), we performed a functional analysis of this region of dMTF-1 both in *Drosophila* and in *S. cerevisiae*.

Transgenic flies expressing wild-type and mutant dMTF-1 genes

To investigate whether the Cys-cluster in *Drosophila* MTF-1 plays any role in copper homeostasis, we generated transgenic flies with constructs in which subsets of cysteines, or all six of them, are substituted by alanines (Figure 1A). As mentioned, *dMTF-1* mutant flies are sensitive not only to excess copper but also to copper depletion (34). This is due to the fact that dMTF-1 activates two sets of genes that are working in opposing conditions, namely, metallothioneins at high copper, and the copper importer *Ctr1B* at times of copper deprivation (35). The sensitivity of *dMTF-1* mutants can be rescued by co-expression of wild-type *dMTF-1* transgene. To examine the role of the cysteine-rich domain, we introduced the mutant constructs *dMTF-1*$^{2C\text{-}2A}$, *dMTF-1*$^{4C\text{-}4A}$ or *dMTF-1*$^{6C\text{-}6A}$ encoding double (C560A/C565A), quadruple (C547A/C549A/C552A/C554A) or complete (C547A/C549A/C552A/C554A/C560A/C565A) alanine substitutions (Figure 1A), into *dMTF-1* mutant flies lacking endogenous dMTF-1 and tested whether these constructs could rescue the sensitivity to either copper supplementation or copper depletion. All of the three *dMTF-1* derivatives are able to rescue the sensitivity to copper starvation as well as the wild-type *dMTF-1* transgene (Figure 1B). This result demonstrates that the wild-type and mutant forms of dMTF-1 are expressed to similar levels since a functional dMTF-1 is required for this.

In contrast, *dMTF-1*$^{2C\text{-}2A}$ and *dMTF-1*$^{4C\text{-}4A}$ could only partially rescue the sensitivity to copper while *dMTF-1* mutant flies expressing *dMTF-1*$^{6C\text{-}6A}$ failed to survive to adulthood in copper supplemented food (Figure 1B). To further understand the molecular mechanism of the copper sensitivity phenotype, we examined the expression of metallothionein A (MtnA) in flies expressing either the wild-type or mutant alleles of *dMTF-1* (Figure 2A). *MtnA* transcript abundance was measured by quantitative S1 nuclease mapping experiments. These data show that under copper stress (100 μM), the wild-type *dMTF-1* transgene strongly activates the transcription of *MtnA* while *dMTF-1*$^{6C\text{-}6A}$ transgene is completely unable to induce *MtnA* transcription. Interestingly, *dMTF-1*$^{4C\text{-}4A}$ and *dMTF-1*$^{2C\text{-}2A}$ transgenes mediate some Cu(I)-induced *MtnA* expression, but to a lesser extent than

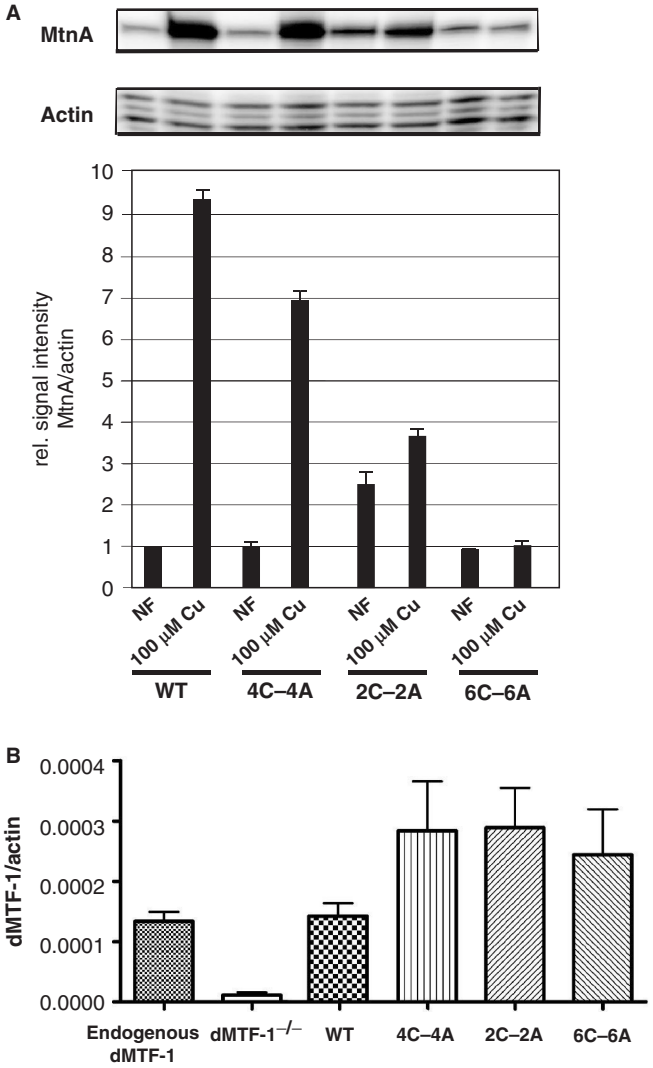

Figure 2. The Cys-rich domain of dMTF-1 is required to activate *MtnA* expression in transgenic flies. (**A**) Total RNA was isolated from transgenic *Drosophila* at the third instar larval stage expressing either a wild-type *dMTF-1*, *dMTF-1*$^{4A\text{-}4A}$, *dMTF-1*$^{2C\text{-}2A}$ or *dMTF-1*$^{6C\text{-}6A}$ allele raised on normal food (NF) or on 100 μM $CuSO_4$ (Cu). MtnA and *actin5c*-specific transcripts were measured by S1 nuclease mapping and are shown as a ratio of transcript abundance. (**B**) *Drosophila* with indicated genotypes was allowed to develop on standard food until third instar larval stage. Total RNA was isolated from larval gut and analyzed by quantitative RT-PCR to quantify transcripts of *dMTF-1*. *Actin-5c* transcripts served as a normalization reference.

wild-type *dMTF-1*. Control experiments reveal that the wild-type and mutant *dMTF-1* transgenes are expressed to similar levels in the larval gut, with the expression of the mutant *dMTF-1* alleles perhaps even slightly (≈2-fold) higher; this and the fact that all transgenes equally confer resistance to copper starvation (see Figure 1B) render unlikely the possibility that the observed phenotypes could be due to insufficient expression of mutant alleles (Figure 2B). Taken together, these data show that the cysteine-rich domain of dMTF-1 is critical for copper-induced transcriptional activation but is clearly dispensable for sensing copper scarcity.

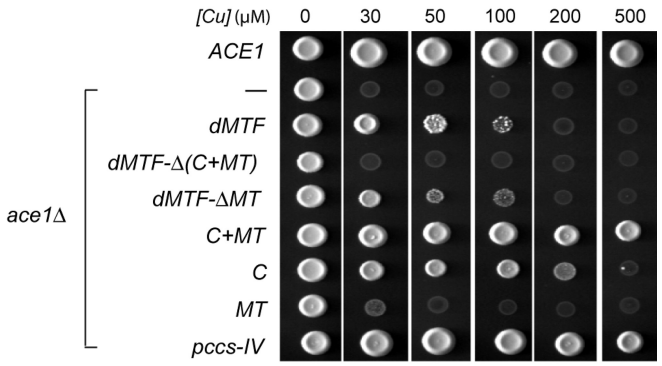

Figure 3. The Cys-rich domain of dMTF-1 protects a Cu-sensitive strain of baker's yeast from the effects of Cu toxicity. *S. cerevisiae* strain DTY59 (*ace1*Δ) was transformed with a plasmid expressing either intact *dMTF-1* (dMTF) or the indicated domain fragments of *dMTF-1* and spotted onto agar plates in a defined medium containing the indicated concentration of $CuSO_4$. A fragment encoding domain IV of the copper chaperone for SOD1 in *S. pombe* Pccs (labeled *pccs-IV*) is a positive control for this experiment. –, empty vector control; *ACE1*, isogenic wild-type strain DTY7 transformed with empty vector.

The cysteine-rich region protects *S. cerevisiae* against copper toxicity

The above results indicate that the cysteine-rich domain plays an essential role in protecting *Drosophila* from copper toxicity by mediating up-regulation of metallothionein genes. In order to assess the importance of this domain relative to other domains in dMTF-1, we have carried out a parallel experiment in a Cu-sensitive *S. cerevisiae* strain, *Δace1*, which lacks the gene for the Cu-dependent activator of *CUP1*, the Cu-binding yeast metallothionein (Figure 3) (38). This strain exhibits severely attenuated survival on Cu-supplemented growth media (first two rows, Figure 3). Expression of *dMTF-1* reverses some of this sensitivity in a manner that absolutely requires the Cys-rich domain (rows 3–5, Figure 3). Interestingly, expression of the entire C-terminal domain of dMTF-1 (C + MT) induces resistance to Cu-toxicity equivalent to that of the MT-like domain of the Cu-chaperone *pccS* of *S. pombe* (45), with most of the protection mediated by the Cys-rich domain itself (rows 6–8, Figure 3).

The cysteine-rich region of dMTF-1 binds four mol•equiv of Cu(I)

We hypothesized that the direct binding of Cu(I) by the sequence encompassing residues 547-565 in dMTF-1 is the basis for copper sensing in cells. To test this, we purified three recombinant dMTF-1 fragments of 131, 81 and 51 amino acids each of which contains the Cys-rich motif, encompassing residues 499-629 (denoted C-dMTF_131), 499-579 (C-dMTF_81) and 529-579 (C-dMTF_51). C-dMTF_81 was chosen for detailed study. Cu(I) titration of C-dMTF_81 (carried out at pH 6.0, 22°C) exhibits intense metal-to-ligand charge transfer absorption (Figure 4A), indicative of coordination to Cys thiolates (44). Similar spectra were obtained for C-dMTF_131 and C-dMTF_51 as well (data not shown). The absorption spectra for C-dMTF_81 saturate at 4 mol•equiv of Cu(I)

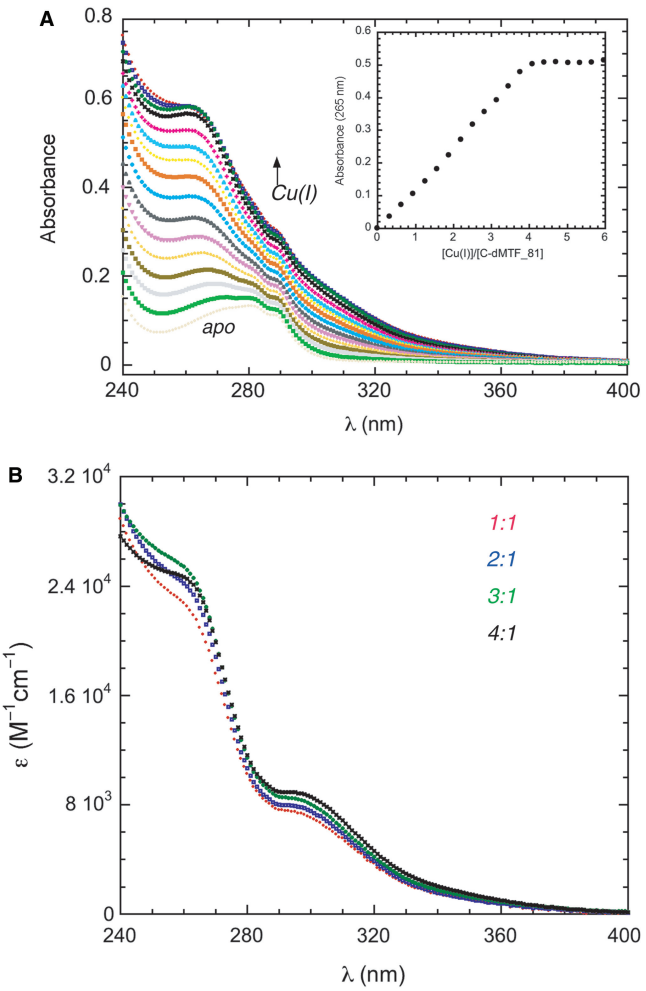

Figure 4. Representative anaerobic titration of C-dMTF_81 with Cu(I). (**A**) Full absorption spectra are shown corrected for dilution, with the apoprotein contribution (*gray curve*) not subtracted. *Inset*, apoprotein-subtracted absorbance at 265 nm from the main body of the figure plotted as a function of Cu(I)/C-d-MTF-1 ratio. (**B**) Apoprotein-subtracted corrected molar absorptivity spectra of Cu(I):C-dMTF_81 mixtures at 1 : 1 (*red*) 2 : 1 (*blue*), 3 : 1 (*green*) and 4 : 1 (*black*) molar ratios. Conditions: 20 μM apo C-dMTF_81, with Cu(I) concentrations ranging from 0.3 to 6.0 molar equivalents, pH 6.0, 22°C.

and the binding is stoichiometric (tight) under these conditions (Figure 4A). Further examination of the absorption spectra at subsaturating amounts of Cu(I) added are consistent with the formation of a single molecular species throughout the course of the titration since molar (per bound Cu(I)) absorptivity spectra of the species formed at 1:1, 2:1, 3:1 and 4:1 Cu(I):C-dMTF_81 molar ratios are identical (Figure 4B) (*vide infra*). These spectra are virtually identical to previously published spectra of Cu_4-Ace1 (46), and are consistent with highly cooperative assembly of $Cu(I)_4$ polynuclear cluster in C-dMTF_81.

C-dMTF_81 also binds Zn(II) ($K_{Zn} \geqslant 10^{10}\,M^{-1}$) and Cd(II) ($K_{Cd} \approx 3 \times 10^6\,M^{-1}$) to form saturating 1:1 complexes under the same solution conditions (Supplementary Figure S1). However, preincubation of C-dMTF_81 with 4 mol•equiv of Zn(II) has virtually no influence on the

Cu(I) binding titration; *i.e.*, Cu(I) still binds stoichiometrically (Supplementary Figure S2A). This suggests that the Cu_4 complex is far more thermodynamically stable than other metallated complexes of C-dMTF_81. Consistent with this, 30 μM C-dMTF_81 is capable of stripping ⩾80% of the Cu(I) from 30 μM Cu(I)-$(BCS)_2$, the latter of which forms with an affinity constant $K_{Cu} \approx 10^{19}\,M^{-1}$(Supplementary Figure S2B). This suggests that the affinity constants for BCS and C-dMTF_81 may be comparable.

Anaerobic titrations like those shown in Figure 4 were also acquired using luminescence spectroscopy (λ_{ex} = 300 nm). The results of a representative titration are shown in Figure 5, with full luminescence emission spectra (Figure 5A) and a plot of the $\lambda_{em,\ 600}$ vs. Cu(I):C-dMTF_81 molar ratio (Figure 5B) shown. These spectra reveal an intensely luminescent species that shows maximum intensity at a molar ratio of 4:1, after which point the intensity sharply decreases. These data reveal that the Cu(I) ions in the Cu_4 polynuclear cluster are significantly shielded from solvent, as has been previously observed for other polynuclear metalloregulatory clusters in *S. cerevisiae* Mac1 and Ace1 (47). Further titration beyond four mol•equiv of Cu(I) results in significant bleaching of the luminescence intensity, which is not observed in an anaerobic optical titration (Figure 2). This suggests that Cu(I) ions that are added beyond saturation induce significant reorganization in the structure, which leads to a less solvent-shielded average environment for the Cu(I) ions. Addition of greater than 4 mol•equiv of Cu(I) to apo-C-dMTF_81 also leads to significant degradation of the ^1H-^{15}N HSQC spectrum (data not shown) consistent with conformational exchange broadening at greater than saturating Cu(I). These complexes may well by oligomeric in nature.

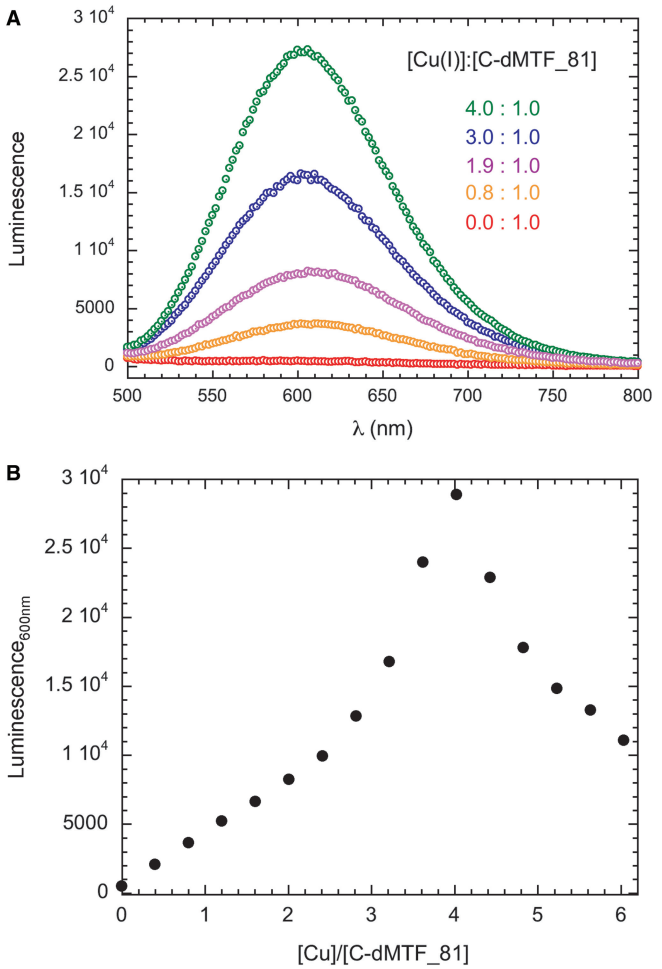

Figure 5. Representative anaerobic titration of apo C-dMTF_81 with Cu(I) as monitored by luminescence spectroscopy. (**A**) Full luminescence spectra (λ_{ex} = 300 nm) acquired as a function of Cu(I):C-dMTF_81 ratio, as indicated. (**B**) Luminescence emission intensity at 600 nm (from panel *A*) plotted a s function of Cu(I):C-dMTF_81 ratio. Conditions: pH 6.0, 25°C.

X-ray absorption spectroscopy reveals a Cu_4-S_6 polynuclear cluster

X-ray absorption spectroscopy was carried out to structurally characterize the copper binding to C-dMTF_81. Figure 6A shows that the Cu K-edge near-edge spectra from Cu(I)-C-dMTF_81 complex prepared with 1.0 and 3.5 mol•equiv of Cu(I) are essentially identical. The peak centered at around 8983 eV, is a 1s→4p transition that is commonly used as a fingerprint for determining the coordination environment of Cu(I) compounds (48). The spectra of the Cu(I)-peptide complexes are very similar to trigonally-coordinated $[Cu_4(SPh)_6]^{2-}$ and distinct from digonally-coordinated $[Cu(SC_{10}H_{12})_2]^{2-}$ (48,49) (Figure 6A), suggesting the former coordination environment in the peptide.

More structural detail is available from analysis of the Cu K-edge extended X-ray absorption fine structure (EXAFS) spectra. Figure 6B and C show the EXAFS, and corresponding Fourier transforms of the Cu(I)-C-dMTF_81 complexes with both 1 : 1 and 3.5 : 1 Cu:peptides, together with best fits. EXAFS curve-fitting parameters are listed in Table SI (Supplementary Material). As with the near-edge spectra, the EXAFS of the two stoichiometries are essentially identical, and gave curve fitting analysis (discussed below) that were also very similar. Two major Fourier transform peaks are observed at ≈2.3 and ≈2.7 Å, and are attributable to Cu—S and Cu ··· Cu interactions, respectively. In agreement with the near-edge spectra (Figure 6A), EXAFS curve fitting indicates three Cu—S at 2.26 Å. Inclusion of lighter scatterers such as N or O resulted in unreasonably small Debye-Waller factors for Cu—S, indicating a sulfur-only $Cu(SR)_3$ coordination. The 2.7 Å Fourier transform peak is best fitted by including two different types of Cu ··· Cu interactions, with two short and one long Cu ··· Cu interactions at 2.70 Å and 2.82 Å, respectively, for 1:1 Cu(I):C-dMTF_81; similar fitted parameters characterize 3.5 : 1 Cu(I):C-dMTF_81 sample as well. The overall similarity of the XAS for both Cu(I):C-dMTF_81 stoichiometries suggests the same Cu center structure and provides direct evidence that C-dMTF-1 binds to Cu(I) cooperatively. Based on the XAS results a Cu_4S_6 polynuclear cluster is proposed to form in Cu(I)-C-dMTF_81, as shown in inset of Figure 6C. MALDI-TOF mass spectroscopy

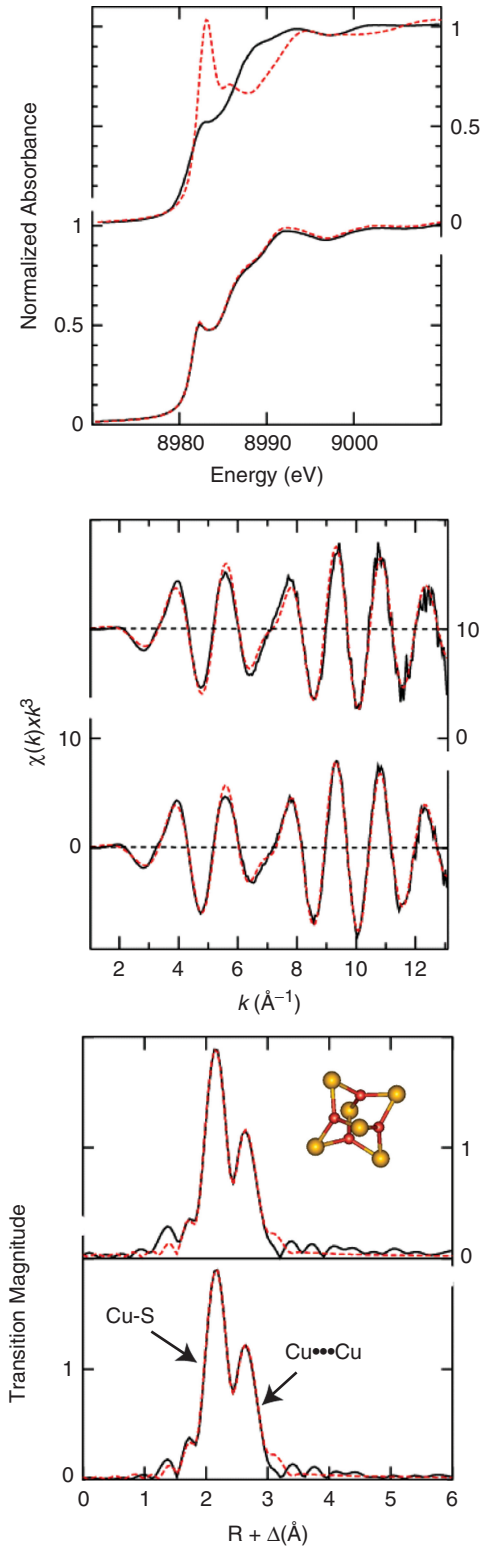

Figure 6. X-ray absorption spectroscopy (XAS) of Cu(I)- C-dMTF_81 complex. (**A**) Cu K-edge near-edge comparison of Cu(I)- C-dMTF_81 complex with two model Cu(I) thiolate compounds. In the upper panel are the trigonal Cu(I) thiolate model $[Cu_4(SPh)_6]^{2-}$ (or $[Cu_4(SR)_6]^{2-}$, black solid line) forming a four-Cu(I) cluster, and the diagonal Cu(I) thiolate model $[Cu(SC_{10}H_{12})_2]^{2-}$ (or $[Cu(SR)_2]^{2-}$, *red dash line*) containing a single Cu(I) ion. The lower panel shows Cu(I)- C-dMTF_81 complex with metal stoichiometries of 1.0 (black solid line) and 4.0 (red dash line), respectively. (**B**) Copper K-edge EXAFS spectra and C) Cu-S phase-corrected EXAFS Fourier Transforms of Cu(I)-C-dMTF_81 complex mixing with 1 mol·equiv. Cu (upper panel) and 4 mol·equiv. Cu (lower panel), respectively. Black solid curves represent the experimental data, while the red dash curves are for best fits with the parameters listed in Supplementary Table S1. The inset shows a structural model representing the proposed metal coordination of the Cu(I)- C-dMTF_81 complex based on the XAS data. The red balls represent copper atoms, while the yellow ones are for sulfur atoms.

of a 1:1 Cu(I):C-dMTF_81 mixture, i.e. identical to the 1:1 sample probed by XAS, as well as a 2:1 Cu(I): C-dMTF_131 mixture, is consistent with this picture, and further suggests that an intramolecular (monomolecular) polynuclear cluster is the dominant conformer in solution (see Supplementary Figure S3).

C-dMTF_81 binds Cu(I) in an all-or-none manner

We first performed a preliminary NMR analysis of 131, 81 and 51 residue fragments of dMTF-1 encompassing residues 499-629 (C-dMTF_131), 499-579 (C-dMTF_81) and 529-579 (C-dMTF_51) by acquiring ^{1}H-^{15}N HSQC and ^{1}H-{^{15}N}heteronuclear NOE (ssNOE) spectra in the presence and absence of Cu(I). The latter experiment carried out with C-dMTF_81 revealed that only ≈27 crosspeaks were characterized by positive ^{1}H-{^{15}N} ssNOE values and were significantly shifted following the addition of 4.0 mol•equiv of Cu(I). This finding is consistent with the idea that Cu(I) folds the region immediately around the Cys cluster with little additional long-range folding evident in these spectra (Supplementary Figures S4-S5); in the absence of Cu(I), all resolvable crosspeaks have strongly negative ssNOE values revealing little or no stable structure in the absence of Cu(I) (spectra not shown). Further evidence for limited and localized Cu-dependent folding is that amide resonances that shift upon addition of Cu(I) have virtually identical chemical shifts in the context of a fusion protein in which 27-residues of dMTF-1 (542-568) are C-terminally appended to protein G B1 domain (GB1) (spectra not shown) (50).

We next used NMR spectroscopy to investigate the cooperativity of Cu_4 cluster formation by acquiring ^{1}H-^{15}N HSQC spectra as a function of Cu(I):C-dMTF_81 molar ratio (Figure 7). These spectra reveal that at subsaturating Cu(I), the spectrum corresponds to a superposition of apo- and Cu_4 conformers with no evidence of a non-native structural intermediate. Quantitation of the crosspeak intensities of selected resonances (Supplementary Figure S6) as a function of Cu(I) loading is fully compatible with scenario, i.e. the intensity of apo-C-dMTF_81 crosspeaks decrease monotonically as Cu_4 crosspeak areas increase. The assembly of the Cu_4 cluster is therefore highly cooperative, a result consistent with the findings by XAS and mass spectrometry, which reveal significant Cu_4 polynuclear cluster upon addition of sub-stoichiometric Cu(I). Despite the highly cooperative Cu-binding by C_dMTF_81, the peptide is characterized by a high degree of internal dynamics, a characteristic not unprecedented from previous studies of Cu- and Zn/Cd-loaded metallothioneins (51).

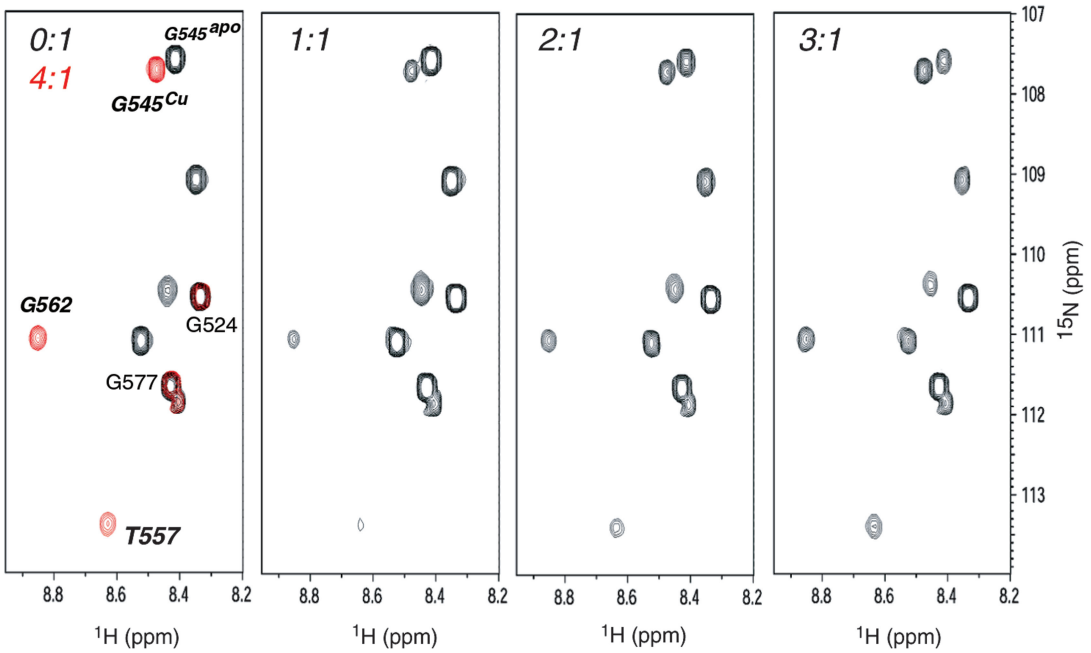

Figure 7. Substoichiometric addition of Cu(I) to apo-C-dMTF_81 results in cooperative assembly of a Cu_4 cluster from the apoprotein. A subregion of ^{1}H-^{15}N HSQC spectrum is shown as a function of Cu(I): C-dMTF_81 molar ratio indicated. Select resonances within the Cys cluster domain are *italicized*, while those outside this region (G524, G577) do not shift upon Cu(I) binding. The three remaining crosspeaks are unassigned apoprotein crosspeaks.

DISCUSSION

MTF-1 of *Drosophila* is capable of activating the transcription of distinct metallothionein genes (*MtnA-D*) in response to several metal ions, including Cu(I), Cd(II) and Zn(II). A recent characterization of transgenic flies carrying deletions of one of the four *Mtn* genes reveals that *MtnA* (the expression of which is studied here) is primarily responsible for protecting flies against exogenous copper load, while $MtnB^{-/-}$ flies are most sensitive to cadmium toxicity. The biological roles of MtnC and MtnD, which are closely related to MtnB, remain enigmatic since flies harboring a deletion of one or both of these genes exhibit near wild-type resistance against Cu and Cd toxicity (37). Binding studies revealed that MtnA is most strongly stabilized by Cu(I) binding, while MtnB binds Cd(II) preferentially over Zn(II) and Cu(I). These findings are generally consistent with the characteristics of flies harboring a metallothionein gene family knockout; these flies are viable and develop normally on standard food, but are highly sensitive to copper and cadmium toxicity. In particular, these experiments establish that MtnA and its regulator MTF-1 are responsible for the intense orange copper-mediated luminescence (when excited in the ultraviolet; see Figure 5) associated with specialized cells from the intestinal tract, termed midgut 'copper cells' (36). These cells likely function as storage depots for excess Cu(I), essentially protecting the organism against the effects of Cu(I)-mediated oxidative stress as well as a source of intracellular copper under conditions of copper deprivation (35). Interestingly, in contrast to mammalian MTF-1, *Drosophila* metallothioneins appear to play only a minor role against zinc toxicity (36). On the other hand, the expression of the zinc efflux transporter ZnT35C, thought to be analogous to the mammalian zinc exporter ZnT1, is strongly induced by Zn in an MTF-1-dependent manner (52).

How a single transcriptional activator, dMTF-1, is capable of up-regulating the expression of specific genes in response to distinct metal ions is unclear. One plausible scenario is that the metal selectivity of gene expression is dictated by the promoter-specific nature of the protein complex containing MTF-1 that mediates a specific transcriptional response. There is some evidence in support of this idea, since when MREs are excised from their context in the *Ctr1B* promoter (which is induced by Cu-scarcity) and placed in a non-native, mini-promoter context, they simply function as activating elements in response to copper overload, just like those derived from metallothionein genes (which are activated upon Cu-overload) (34). Along this vein of thought, MTF-1 might function as a promoter-specific adaptor molecule, in which the Zn(II)-bound zinc fingers mediate a direct interaction with the MRE, and another domain of the molecule mediates a Cu- or Cd- or Zn-specific complex with a putative co-activator or co-repressor. The foundational tenet of this hypothesis is that MTF-1 should be capable of forming complexes with Cu or Cd/Zn, with the distinct structures of each coordination complex (Cu vs. Zn/Cd) required to mediate metal-specific protein-protein interactions.

In the work presented here, we show that a C-terminal Cys-cluster of dMTF-1 encompassing six closely spaced cysteines forms a very stable, highly cooperative brightly luminescent Cu_4-S_6 polynuclear cluster. This cluster is essential for dMTF-1 to drive the expression of its target

gene *MtnA*, because a complete Cys substitution (6C-6A) in dMTF-1 abolishes its activity under copper stress and keeps the *MtnA* gene uninduced. Such a defect at molecular level results in a copper sensitive phenotype of mutant *Drosophila*. Partial alanine substitution of two (2C-2A) or four (4C-4A) of the Cu(I)-liganding cysteines also results in a severely attenuated survival index; this suggests that formation of the Cu_4-L_6 (L = ligand) complex optimally protects flies against copper toxicity by inducing *MtnA* expression. This short 19-amino acid domain is necessary and sufficient to bind four mol•equiv of Cu tightly and stoichiometrically *in vitro* and *in vivo*, the latter measured by examining the viability of Cu-sensitive *S. cerevisiae* strain on Cu-supplemented media.

Strikingly, the cysteine-rich domain of dMTF-1 is reminiscent of Cu-sensing domains of other Cu-regulators from lower eukaryotes, including Mac1 and Ace 1 from *S. cerevisiae*, Cuf1 from the fission yeast *S. pombe* (40), GRISEA from *Podospora anserina* (39), and Amt1 from *Candida glabrata* (41), in the complete absence of amino acid sequence homology. Spectroscopic studies of Amt1, Mac and Ace1 reveal that each forms intensely luminescent tetranuclear Cu_4•L_6 'cage-like' clusters containing trigonally coordinated solvent-shielded Cu(I) ions, with significant Cu•••Cu interactions, that either stimulate (Ace1, Amt1) or inhibit (Mac1) promoter DNA binding and/or transcriptional activation (41,47). A characteristic feature of the Cu complexes formed by Amt1, Ace and Mac1 is a short 2.7 Å Cu-Cu distance, also found here for dMTF-1 (41,47). Extensive molecular genetic studies have been carried out on *S. cerevisiae* Mac1, and these experiments are consistent with a model in which the Cu-binding domain forms a direct intramolecular protein–protein interaction with the N-terminal DNA-binding and nearby transactivation domain that allosterically blocks Mac1 function at multiple levels (53–55). Since Mac1 regulates the expression of the two high affinity Cu-importers CTR1 and CTR3, Cu-replete cells turn off the transcriptional activity of Mac1 in a Cu-dependent manner. In contrast, Cu-binding to both Ace1 and Amt1 strongly activates binding to the CuREs (copper response elements) positioned upstream of the genes encoding two metallothioneins, *CUP1* and *CRS5*, and superoxide dismutase *SOD1*. It seems plausible that Cu-binding to the C-terminal domain in dMTF-1 might unmask a critical transcription activation domain that allows the recruitment of TFIID to the promoter (32) or perhaps the chromatin remodeling enzymes, Swi5/Snf and Gcn5, as has been demonstrated for *C. glabrata* Amt1 (56).

The Cu-regulatory complexes formed by dMTF-1 and yeast transcriptional activators contrast sharply with those found in known copper metalloregulatory proteins in prokaryotes, which form either digonal (57), mononuclear trigonal planar (44), or binuclear Cu_2•S_4 coordination complexes (2,58). Unlike each of these systems which are highly specific for Cu(I) (and its structural surrogate Ag(I)) (46), the intrinsic metal specificity of the metal sensing domain of dMTF-1 may well be relaxed since dMTF-1 has to bind and metalloregulate gene expression from a variety of promoters in response to a number of different metal ions, including Cd(II) and Zn(II). A direct

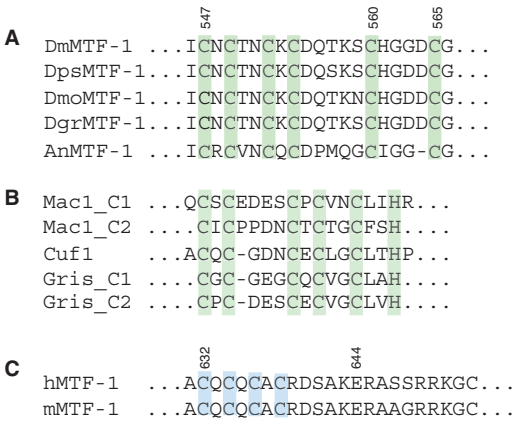

Figure 8. Cysteine clusters of metalloregulatory transcription factors. (**A**) Conservation of the Cys-rich region in MTF-1 of *Drosophilidae* and a mosquito. Dm, *Drosophila melanogaster*; Dps, *Drosophila pseudoobscura*; Dmo, *Drosophila mojavensis*; Dgr, *Drosophila grimshawi*; An, *Anopheles gambiae* (23). (**B**) Cys-rich domains of yeasts and a filamentous fungus (39,40,47). Mac1 and Cuf1, copper-regulated transcription factors of baker's yeast (*S. cerevisiae*) and fission yeast (*S. pombe*), respectively. Grisea, copper-responsive transcription factor of the fungus *Podospora anserina*. (**C**) Tetracysteine cluster of human and mouse MTF-1, required for transcriptional response to zinc and cadmium load (9,23).

role of the Cys-cluster in sensing both Cd(II) and Zn(II) would require that the Cys-cluster of dMTF-1 bind these metal ions as well. In fact, the Cys cluster in dMTF-1 forms stoichiometric 1:1 complexes with both Cd(II) and Zn(II), rather than a polynuclear cluster; however, Cu(I) easily outcompetes Zn(II), with Zn(II) binding considerably more tightly than Cd(II) (Supplementary Figure S1). It seems likely then that Cu(I) is the 'cognate' metal and others may have to be recruited under specialized intracellular conditions at specific promoters. It will be interesting to determine the degree to which inactivation of the Cys-cluster by mutagenesis influences the metal-selectivity and inducibility at other promoters, in particular those that respond to other metal ions. In any case, under the chelator conditions tested, the cysteine mutants of dMTF-1 were no more sensitive to copper starvation than wild-type flies (Figure 1B). This likely indicates that the regulation of the *Ctr1B* copper importer gene by dMTF-1 (34) involves protein domain(s) other than the cysteine cluster characterized here.

In conclusion, we have identified a novel Cu-binding domain in dMTF-1 derived from a cluster of six cysteines that is required to regulate metallothionein expression in transgenic flies in response to toxic intracellular levels of Cu(I). The structural features of this Cu-sensing domain while reminiscent of those previously identified in a number of fungal copper regulators, occurs in the absence of significant sequence homology and is therefore consistent with convergent evolution (Figure 8). These findings reveal a functional conservation of Cu homeostasis and detoxification from fungi to flies, with the added twist that just one transcription factor, dMTF-1, which must have evolved independently of the fungal regulators, handles both the uptake and detoxification arms of the Cu homeostasis system in *Drosophila* (34).

Ongoing studies in our laboratories are directed toward understanding the molecular mechanism of differential sensing and regulation performed by MTF-1 in response to a variety of inducers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Short Communication

Mercury and cadmium trigger expression of the copper importer Ctr1B, which enables *Drosophila* to thrive on heavy metal-loaded food

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Abstract

Organisms from insects to mammals respond to heavy metal load (copper, zinc, cadmium, and mercury) by activating the transcription factor metal-responsive transcription factor 1 (MTF-1). MTF-1 binds to short DNA sequence motifs, termed metal response elements, and boosts transcription of a number of genes, notably those for metallothioneins. In *Drosophila*, MTF-1 somewhat counter-intuitively also activates transcription of a copper importer gene (*Ctr1B*) in response to copper starvation. Here, we report that mutant flies lacking Ctr1B are extremely sensitive to cadmium and mercury treatment, but can be rescued by excess copper in the food. We thus propose that copper, by competing for binding sites on cellular proteins, alleviates the toxic effects of mercury and cadmium. Such a scenario also explains a seemingly fortuitous metal response, namely, that cadmium and mercury strongly induce the expression of a Ctr1B reporter gene. Thus, the transcription enhancer/promoter region of the Ctr1B copper importer gene is subject to three modes of regulation. All of them depend on MTF-1 and all make biological sense, namely, (i) induction by copper starvation, (ii) repression by copper abundance, and (iii), as shown here, induction by cadmium or mercury at normal copper supply.

Keywords: copper transporter; heavy metal toxicity; metal homeostasis; MTF-1; non-essential metals.

Copper is an essential component of several important enzymes involved in cellular processes, such as respiration, oxidative stress protection, pigmentation, and iron homeostasis (Balamurugan and Schaffner, 2006; Kim et al., 2008). At the same time, copper poses a threat to the organism, especially as it can catalyze the generation of reactive oxygen species (ROS) via the Fenton reaction (Halliwell and Gutteridge, 1990; Puig and Thiele, 2002). Eukaryotic organisms from yeast to humans use elaborate systems to regulate copper homeostasis, consisting of copper importers, copper chaperones, inducible transcription factors, small metal binding proteins called metallothioneins, and copper exporters (O'Halloran and Culotta, 2000; Puig and Thiele, 2002; Mercer and LIanos, 2003; Rutherford and Bird, 2004; Balamurugan et al., 2007). High affinity copper transporters were identified in yeast (Ctr1, 2, and 3), mammals (Ctr1 and 2), and in *Drosophila* (Ctr1A, B, and C) (Dancis et al., 1994; Lee et al., 2000; Zhou et al., 2003). Null mutants of *Ctr1* in the mouse and of *Ctr1A* in *Drosophila* are lethal, while a mutation of *Drosophila Ctr1B* results in a pigmentation defect and lethality under conditions of copper scarcity (Kuo et al., 2001; Lee et al., 2001; Zhou et al., 2003; Selvaraj et al., 2005; Turski and Thiele, 2007).

From insects to mammals, the major transcriptional regulator handling heavy metal toxicity is metal-responsive transcription factor 1 (MTF-1, also referred to as metal response element binding transcription factor 1) (Radtke et al., 1993; Otsuka et al., 2000; Andrews, 2001; Giedroc et al., 2001; Lichtlen and Schaffner, 2001; Egli et al., 2003; Balamurugan et al., 2004). MTF-1 is a zinc finger protein of some 700 amino acids that recognizes short DNA sequence motifs termed metal response elements (MREs) in the enhancer/promoter region of target genes, notably metallothionein genes (Stuart et al., 1984; Wang et al., 2004). MTF-1 is activated by heavy metal load and, somewhat counter-intuitively, also has a role during copper starvation, whereby it induces transcription of the *Ctr1B* copper importer in *Drosophila* (Selvaraj et al., 2005). Conversely, copper load results in an MTF-1 dependent inhibition of *Ctr1B* expression (Zhou et al., 2003; Selvaraj et al., 2005). In follow-up experiments presented here we have made the observation that *Ctr1B* expression is indifferent to zinc status, but strongly induced by exposure of *Drosophila* larvae to the non-essential, toxic metals mercury and cadmium. What initially appeared as a fortuitous response to rare non-essential metals turned out to be of vital importance: flies lacking *Ctr1B* are extremely sensitive to mercury and cadmium, and toxicity can be alleviated by feeding the mutants a high-copper diet. Thus, Ctr1B counteracts mercury and cadmium toxicity by importing copper, which presumably acts as a less deleterious competitor for metal binding sites within the cell.

As a reporter gene to study expression and regulation of the Ctr1B copper importer, we used a Ctr1B transgene whose complete coding sequence was fused to the one of green fluorescent protein (eGFP) (Figure 1A). This construct, designated AH3, reproduced the regulation of the endogenous Ctr1B gene as determined by transcript mapping, namely, high expression upon copper starvation but low expression upon copper load (Zhou et al., 2003; Selvaraj et al., 2005). Similar to the endogenous

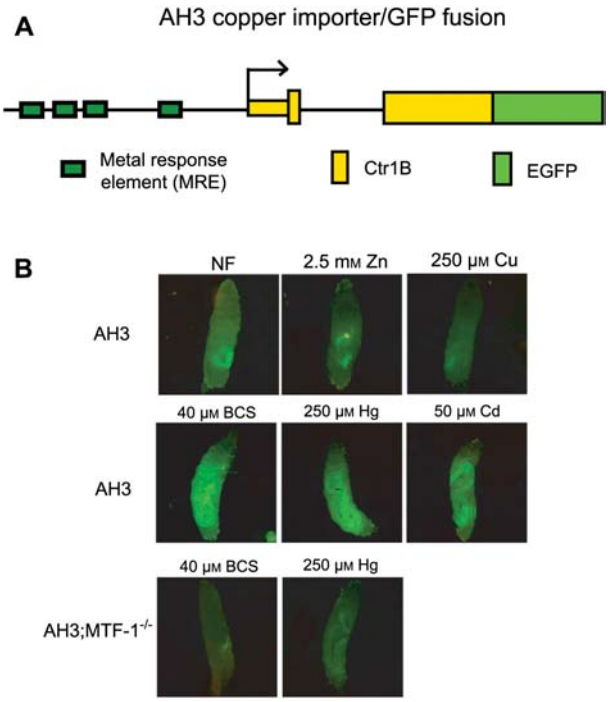

Figure 1 The Ctr1B copper importer gene is not only activated by copper starvation but also by the non-essential, toxic metals mercury, and cadmium.
(A) Transgenic Ctr1B reporter gene ('AH3'; Selvaraj et al., 2005). In this construct, genomic DNA including the upstream enhancer with multiple metal response elements (MREs, shown as green boxes) and the complete 5′UTR and coding sequence of Ctr1B is fused to the coding sequence of green fluorescent protein (eGFP). This indicator transgene, which still functions as a copper importer, was integrated via P-element transformation into the genome of *Drosophila*. (B) Expression of the Ctr1B reporter gene is activated by the non-essential metals mercury and cadmium. Wild-type flies or flies mutant for transcription factor MTF-1 (*dMTF-1*$^{140\text{-}1R}$; Egli et al., 2003) were allowed to lay eggs in culture tubes containing normal food (NF) or food with the indicated supplements. The typical intermediate level expression was not affected by zinc, but inhibited by copper load (first row). Ctr1B expression was induced by copper starvation (copper chelator BCS; Zhou et al., 2003; Selvaraj et al., 2005), and most notably also induced by cadmium or mercury ions (second row). This activation was dependent on the MRE-binding transcription factor MTF-1 (third row and not shown).
Methods: all fly strains used for this study were Oregon R with a yw background. Flies were grown from the egg stage in the indicated type of food (for simplicity, the terms or symbols copper, cadmium, zinc, and mercury refer to Cu^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+}, respectively) and the resulting third instar larvae were taken for analysis. Images were taken with a Leica MZ FLIII fluorescence stereomicroscope (●●Name of manufacturer, name of city, state and country? ●●) and a Nikon Coolpix950 digital camera (●●Name of manufacturer, name of city, state and country? ●●) for whole larvae.

gene, regulated expression of this transgene depended on an upstream cluster of metal response elements (MREs) and on the presence of MTF-1, the cognate transcription factor: in MTF-1 null mutant larvae, expression remained low and was unaffected by copper concentration. In a complementary experiment we also included a treatment with other metals. While Ctr1B was indifferent to zinc supplement, expression was strongly induced by mercury or cadmium, two non-essential, notoriously

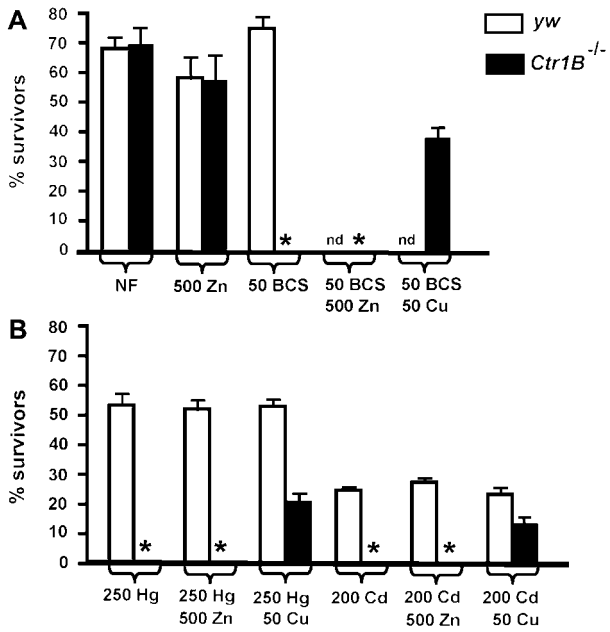

Figure 2 Sensitivity of Ctr1B mutants to cadmium and mercury is rescued by copper supplementation.
Ctr1B null mutants (Zhou et al., 2003) were compared to 'wild type' (yw) flies. The percentage of laid eggs which develop to adulthood (enclosure from pupal stage) in the various types of food is indicated in the ordinate. (A) Control experiments. Most *Drosophila* eggs develop to adulthood in normal food (NF), with or without containing functional Ctr1B (white and black bars, respectively); mutants are killed by a moderate concentration of copper chelator, an effect which is rescued by copper, but not by zinc, supplement. Note that for simplicity all micromolar concentrations are indicated numerically (e.g., 500=500 μM). (B) Ctr1B null mutants are highly sensitive to mercury and cadmium but can be rescued to a large extent by copper supplement. Copper was supplemented simultaneously with other metals (cadmium, mercury). *Indicates no survivors; nd, not determined, but established previously to allow for survival of wild-type (yw) *Drosophila* (Egli et al., 2003 and data not shown).

toxic environmental pollutants (Figure 1B). Based on the fact that induction depended on MTF-1, we conclude that regulation of the Ctr1B copper importer gene also involves its upstream cluster of MREs, but with an effect opposite to that of copper. Initially, these results were interpreted as fortuitous response of fly larvae to toxic heavy metals which presumably are not regularly encountered in the wild. This view had to be revised when we also tested a *Drosophila* mutant lacking a functional Ctr1B. This fly strain develops well in normal food (Cu content, 2–5 μM), but is sensitive to copper depletion. Unexpectedly, the Ctr1B mutant was also sensitive to the non-essential metals mercury and cadmium, while there was no sensitivity with zinc load. [Zinc homeostasis in higher eukaryotes including *Drosophila* is handled efficiently by a different set of transport proteins, notably ZIP-type importers and ZnT-type exporters (Mathews et al., 2005; Yepiskoposyan et al., 2006).] The sensitivity to cadmium and mercury was all the more surprising in light of the fact that *Drosophila* is quite metal-resistant and readily develops to adulthood in food containing either 200 μM cadmium or 250 μM mercury. Under these latter conditions, not a single larva of the Ctr1B mutant survived (Figure 2 and data not shown).

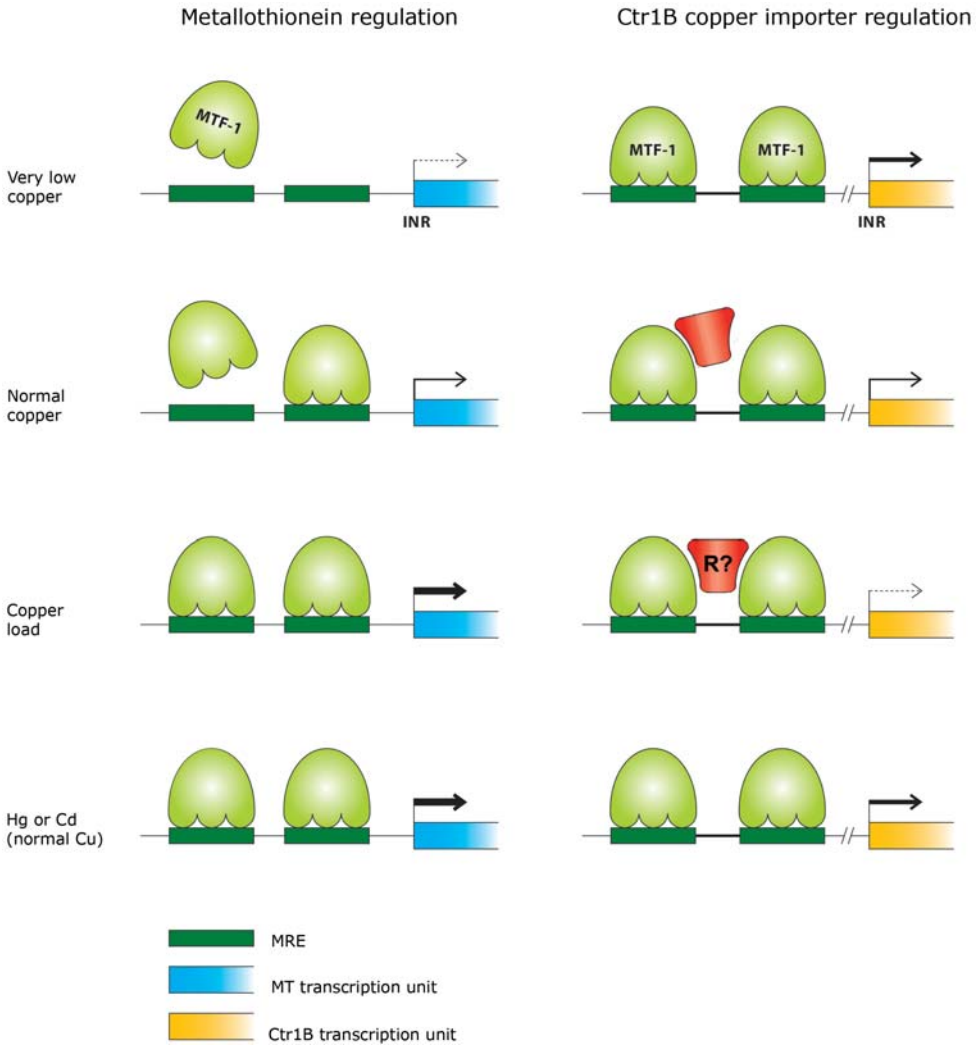

Figure 3 Model of differential response of metallothionein and Ctr1B genes to distortions of physiological metal concentrations. Transcriptional regulation of metallothionein and of Ctr1B copper importer genes is schematically shown on the left and right hand side, respectively. Regulation of both depends on metal responsive transcription factor (MTF-1). Note that with copper, the two genes respond oppositely to abundance and depletion, indicated by thickness of arrows at the initiation region for transcription (INR): copper load has a negative effect on Ctr1B transcription by inducing an unfavorable MTF-1 conformation, or association with a (hypothetical, copper-specific) repressor (R?). The unique property of MTF-1-mediated repression is dependent on the specific spacing of metal response elements (MREs) (Selvaraj et al., 2005). By contrast, Ctr1B is induced, concordantly to metallothionein, by the toxic metals cadmium or mercury as a cellular defense reaction.

To test whether copper homeostasis was somehow linked to the ability of *Drosophila* to cope with cadmium or mercury, we repeated the experiments whereby half of the food samples were also supplemented with 50 μM copper salt. Indeed, while the sensitivity of the *Ctr1B* mutant to mercury and cadmium was reproduced, supplementation with copper largely or completely rescued the lethality phenotype. By contrast, food supplementation with zinc failed to alleviate the deleterious effects of mercury or cadmium. From these results, we conclude that copper plays a central role in counteracting the toxicity of the two non-essential metals. The extra copper imported by the larvae probably helps to fence off mercury and cadmium from binding to, and thereby inactivating, some essential protein(s). Alternatively, copper might exert its protective effect via a particularly strong and/or rapid induction of mRNAs encoding metallothioneins, the major heavy metal-scavenging proteins. We consider this possibility less likely, because both cadmium and mercury are potent inducers of metallothionein gene transcription (Zhang et al., 2001; Balamurugan et al., 2004; Egli et al., 2006). Whatever the exact mechanism, the seemingly awkward induction of *Ctr1B* upon treatment with mercury or cadmium (Figure 1B) is in fact biologically meaningful: there must be a sensing of the toxic effects and, mediated by MTF-1, a compensatory activation of the copper importer. It will be a challenge to find out why the upstream enhancer region of *Ctr1B*, including the MRE cluster, is repressed by copper, but responds to mercury and cadmium in a manner indistinguishable from metallothionein gene promoters, which are activated via MTF-1 by a variety of metals, notably copper, zinc, cadmium, and mercury. One clue to this enigma is the spacing of metal response elements in the *Ctr1B* promoter. Spacing of three MREs is conserved among drosophilids and functionally important, while the MREs in metallothionein promoters mediate a metal response independently of their spacing. We have shown

before that a synthetic regulatory sequence, consisting of the MREs of the *Ctr1B* upstream enhancer but with reduced spacing between them, behaves like a metallothionein promoter in that it becomes responsive to copper, instead of being repressed (Selvaraj et al., 2005). It is tempting to speculate that the combination of MREs and their spacing is generating a situation where copper, unlike mercury and cadmium, induces an unfavorable conformation of MTF-1 or a recruitment of a (hypothetical) repressor to the regulatory DNA of the *Ctr1B* gene (see model in Figure 3).

What originally appeared to be an erratic response to toxic agents, which might rarely be a threat to wild-living *Drosophila*, turned out to be a biologically meaningful, life-saving response. This response is further testimony to a surprising ability of living systems to cope with situations which are not part of their daily encountered, perhaps not even once-per-generation encountered, environmental conditions.

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Effects of metal homeostasis and oxidative stress on Aß42 induced apoptosis in a *Drosophila* model for Alzheimer's disease

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder with increasing prevalence among old people characterized by amyloid plaques and neurofibrillary tangles in the brain (Goedert and Spillantini, 2006). Until now there is no cure for it and its molecular basis is only partially understood. The main component of amyloid plaques, amyloid ß peptide (Aß), is processed from ß-amyloid precursor protein (APP) by proteolytic cleavage (Kang 1987). Aß occurs in various lengths, from 35aa to 49aa. Among these, Aß42 is the most aggregation-prone and neurotoxic form. The monomer form of Aß is not toxic (Selkoe 2008) or at least less toxic than aggregation-mediated multimers which are neurotoxic. *In vitro* studies have revealed that binding of Cu(II) or Zn(II) strongly promotes the aggregation of Aß (Bush et al., 1994; Atwood et al., 1998). Accordingly, metal chelators can dissolve the Aß plaques from postmortem AD brains. Studies have shown that His 6, His 13 and His 14 are crucial for Aß to bind Cu(II) and Zn(II) (Atwood et al., 2000; Danielsson et al., 2007). In the amyloid plaques, copper and zinc are highly concentrated (Lovell et al., 1998). However, AD patients show decreased copper levels in the cortex (Maurer et al., 2000; Cottrell et al., 2001). Dietary Cu supplementation was shown to restore the activity of Cu/Zn SOD in a transgenic mouse model of AD (Bayer et al., 2003). Aß-copper complexes were shown *in vitro* to generate reactive oxygen species (ROS) via a Fenton-like reaction (Huang et al. 1999). The fact that Aß42 has a higher capability to generate ROS than Aß40 may at least in part explain why Aß42 is more neurotoxic. Hence, the interactions among Aß, metals and oxidative stress may be at the center of the pathogenesis of AD. However, the question how these factors would influence the metabolism of Aß *in vivo* has not been extensively investigated. This has prompted us to explore the effects of metals and oxidative stress on toxicity of Aß42 using the fruit fly *Drosophila* as a model. Our data demonstrate that copper, zinc and oxidative stress enhance the toxicity of Aß while metal chelators, metal scavenger proteins and antioxidant proteins attenuate Aß-mediated phenotypes. Thus, results from the present study suggest antioxidant therapies and metal chelation as potential strategies.

Results

Zn and copper strongly enhance the toxicity of Aß42

To investigate the toxicity of Aß peptides *in vivo*, we generated transgenic flies expressing Aß peptides. Aß42 peptide expression levels and the processing of the signal peptide in the transgenic flies were examined by Western blot and mass spectrometry. When Aß42 is expressed under the control of an eye-specific driver (GMR-Gal4), the signal peptide is correctly processed (Fig 1). Expression of Aß peptides in the eye causes a range of abnormalities. The severity of such eye phenotypes correlates with the expression levels of Aß42 peptides as well as the age of the flies, i.e., when the expression level is higher or the flies are older, the eye phenotypes get stronger (Fig 2).

To test the effect of zinc and copper on Aß toxicity, flies were treated with food containing high zinc or high copper concentrations. Such treatments consistently enhanced the eye distortion phenotype (Fig 3). To check whether the effect of zinc and copper on Aß toxicity is due to direct binding of zinc and copper to Aß peptide, copper/zinc binding sites (His 6,13 and 14) were mutated in Aß42. Such a mutant form of Aß42 causes only a mild phenotype, which cannot be enhanced by zinc anymore (Fig 3), unlike the situation with wild type Aß where zinc/copper chelators strongly ameliorate the toxicity (Fig 4).

MTF-1 and metallothioneins inhibit Aß toxicity

MTF-1 is a key regulator of metal homeostasis and metal detoxification in *Drosophila* (reference). While the lack of MTF-1 renders flies sensitive to metals, overexpression of MTF-1 gives enhanced protection against copper stress (Balamurugan et al., 2007). In our model system, expression of MTF-1 or one of its target genes, metallothionein A (MtnA), strongly reduced Aß toxicity (Fig 5). On the other hand, when Aß was expressed in a MTF-1 mutant background, it became more toxic for the eye tissue. In humans, metallothionein 3 (MT3) is the most abundant metallotionein in the brain. A human MT3 transgene co-expressed with Aß indeed suppresses Aß mediated phenotypes (Fig 5).

Oxidative stress and Aß toxicity

To investigate how oxidative stress affects Aß toxicity, we treated Aß expressing flies with hydrogen peroxide. Our results reveal that hydrogen peroxide at a dosage that does not affect wild type flies strongly enhances the Aß-mediated phenotypes (Fig 6). Since oxidative stress increased Aß toxicity, we reasoned that antioxidant genes might prevent Aß toxicity. Indeed, we found that a transgene encoding glutamate-cysteine ligase (GCL), the key enzyme for glutathione synthesis, is able to ameliorate Aß toxicity (Fig 6).

Aß induces apoptosis

To cast more light on the mechanism of Aß-induced cell death in our *Drosophila* model, we suppressed apoptosis by testing either a small deletion DfH99 or overexpression of p35 protein. DfH99 deletes three pro-apoptotic genes, namely *hid, grim* and *reaper*. In homozygous state it is lethal, but even in heterozygous flies programmed cell death is strongly reduced. p35 is a caspase-inhibitory protein derived from baculovirus. Our results show that both the expression of p35 or DfH99 in heterozygous state decrease the level of cell death induced by Aß42 (Fig 7). The fact that eye degeneration is not prevented completely could indicate that inhibition of apoptosis is incomplete in both cases or that mechanisms other than apoptosis also contribute to Aß-induced cell death.

Discussion

Aß peptides are widely regarded as the primary culprits in Alzheimer's disease (Selkoe 2001). There is compelling evidence that Aß exerts neurotoxicity; however, it is not fully understood what factors affect Aß toxicity in a living organism. Over the last years, *Drosophila* models for Alzheimer's disease were developed (Finelli et al., 2004; Greeve et al., 2004; Crowther et al., 2005). Work from these groups has shown that *Drosophila* recapitulates several pathological features also seen in human AD. We took the advantage of a *Drosophila* model system for AD to test various genetic and environmental factors that may affect Aß toxicity.

In the present study, we demonstrate that elevated zinc and copper levels enhance Aß toxicity. Support for this observation comes from the finding that mutations of the copper/zinc binding sites on Aß peptide block the enhancing effect of copper and zinc on its toxicity. A previous study has revealed that chelation of copper reduces Aß-mediated H_2O_2 generation and oxidative damage (Huang et al. 1999). Recently, metal chelators were developed as a treatment for AD. Among them, clioquinol (CQ) was shown to decrease Aß deposition in Tg2576 transgenic mice (Cherny et al. 2001) and have beneficial effects in a phase II clinical trial. Although the development of CQ discontinued because of its side effects, a new and safer derivative, PBT2, is under investigation. We also tested a specific type of chelators developed by Dpharm Inc. designated membrane activated chelators (MACs). MACs avoid the problems that could be caused by general chelation of metals and were shown to reduce amyloid pathology in Tg2576 mice (Lee et al. 2004). Using our model system, we also performed a small-scale screen to search for more effective MACs (unpublished data). Our results reveal that MACs can efficiently prevent the deleterious influence of copper and zinc on Aß. However, chelators may be beneficial in other means. For example, AD patients have copper deficiency in their brain and it has been suggested that CQ functions as a copper-carrier to effectively increase intracellular copper levels. Therefore, our results support the concept of using specific metal chelators for the treatment of Alzheimer's disease.

In addition to metal chelators, we found that expression of the key regulator of metal homeostasis, dMTF-1, and the human and *Drosophila*

metallothioneins are capable of reducing Aß phenotypes. This is consistent with the previous finding that metallothioneins have neuroprotective functions (Pankowa 2006).

A large body of evidence indicates that oxidative stress contributes to AD. AD patients have higher concentrations of oxidative stress markers than age-matched controls (Markesbery et al. 1998). There is evidence that Aß generates reactive oxygen species (ROS), notably hydrogen peroxide and hydroxyl radical, especially when it binds to redox-active metals (Huang et al. 1999). In our model system, increased levels of oxidative stress correlate with stronger Aß phenotypes. We also found that overexpression of glutamate-cysteine ligase (GCL), an essential protein complex for glutathione production, is able to reduce Aß mediated cell death. Together with metallothioneins, these genes could be candidates for gene therapy to treat AD.

Alzheimer's disease is a multifactorial complex disorder. Focusing on Aß42 and investigating its properties *in vivo* will shed light on the molecular mechanism of AD. Our study may also serve as a basis for designing novel genetic or pharmacological therapies to prevent, or at least ameliorate, AD pathogenesis.

Materials and methods

Fly culture

1 liter of standard fly food was composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar, and 15 ml anti-fungal agent nipagin (15% in ethanol, m/v). For experiments, food was supplemented with $CuSO_4$ or ZnCl2 or bathocuproinedisulfonate (BCS) disodium salt hydrate (Sigma-Aldrich No. 14,662-5) or membrane activated chelators (MACs) to the indicated concentrations. BCS is a specific copper chelator used to deplete copper in the food. MACs (DP-109, DP-736, DP-725, DP-694 and DP-460) are chelators for copper and zinc ions. Flies were raised at 25ºC and 65% humidity.

Plasmids and fly transformation

The Aß42 coding sequence with the signal peptide sequence of Drosophila hedgehog protein was cloned into the P element transformation vector pUAST. Fly transformation was done in the standard way as previously described. Resulting transgenic fly strains that were used in this work were UAS-Aß42S3 and UAS-AßS7. The phage C31 site-specific integration system was used to introduce sequences coding for Aß42 wild type or Aß42 with 3 His->Arg mutations into the same locus. Aß42 wild type sequence and Aß42 with His 6, 13 and 14 mutated into Arg were cloned into a pattB vector containing the 5XUAS enhancer/promoter. Plasmids were introduced into flies via an AttP landing site (line ZH-86Fb). The resulting transgenic flies are designated UAS-Aß-attB and UAS-Aß-3HR-attB.

Fly stocks

The following stocks were used in this work:
y w; + ; +
y w; +; actin-Gal4/TM3,y+
y w; +; GMR-Gal4
y w; UAS-Aß42S3

y w; UAS-Aß42S7
y w; UAS-Aß42N7
y w; UAS-Aß42-attB
y w; UAS-Aß42-3HR-attB
y w; UAS-Aß42S3 ; GMR-Gal4
y w; +; UAS-MTF-1/TM6B,y+
y w; +; UAS-MtnA/TM6B,y+
y w; +; UAS-hMT3
w; +; UAS-p35
UAS-GCLc
UAS-GCLm
Df(3L)H99

Western blot and Mass spectrometry

Protein was isolated from control flies and Aß42 expressing flies with RIPA buffer (50 mM Tris-HCl/ 0.5% sodium deoxycholate/ 1% NP-40/150 mM sodium cloride/ 1% SDS). G-Sepharose beads were preincubated with WO-2 antibody before being used for immunoprecipitation of Aß peptides from the protein lysate. After immoprecipitation, samples were loaded on a Novex 10%-20% Tricine gradient gel. For Maldi-MS, after immunoprecipitation, samples were washed twice with PBS and then washed twice with 50% ammonium acetate. Elution was done with 25% ammonium hydroxide. Maldi-MS was performed as previously described.

Eye section

5-day-old flies were collected and eye sections were analyzed with a Leica Leitz DMRB fluorescence stereomicroscope as previously described. Images were taken with a Zeiss Axiocam.

Survival assay

Flies that carry a UAS-Aß42S7 transgene were crossed with *actin-Gal4/TM3,y+* flies on standard food or on food containing either Cu, Zn, DP-109 or BCS. From the cross, two types of progeny could be obtained:

Progeny (A), flies that were expressing Aß42; progeny (B), flies that were not expressing Aß42. The survival index (Is) was calculated as follows: Is=2A/(A+B).

Locomotion assay

Locomotor activity was measured with a negative geotaxis assay. Groups of female flies were transferred into empty 95 x 27 mm glass tubes which were marked with a horizontal line 7.5 cm above the bottom. Flies were gently shaken down to the bottom of the vial and after 10 s the number of flies that had climbed beyond the 7.5 cm mark was recorded. All the studies were performed under standard light conditions.

Conflict of interest statement

None declared.

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Figures

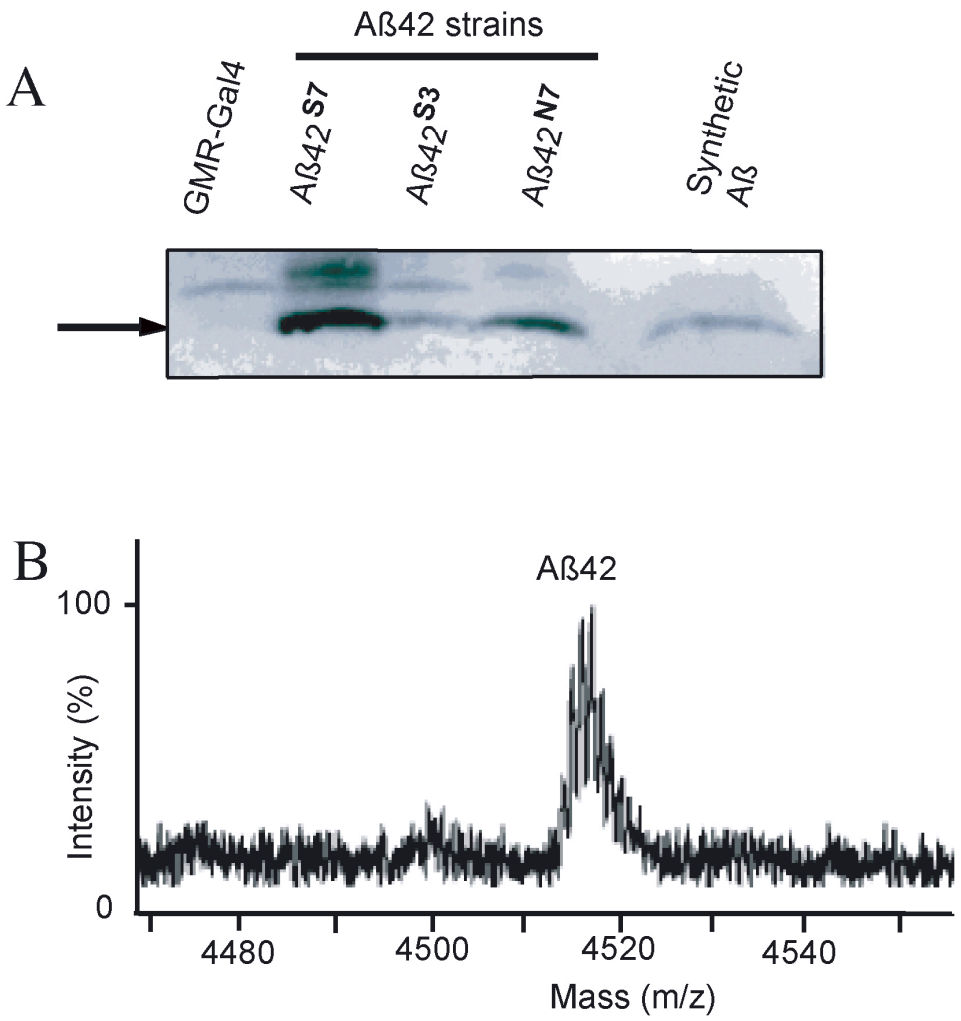


Fig 1. Expression of Aß42 in Drosophila

A) Western blot with antibody for Aß (WO-2) to detect Aß42 in transgenic fly strains. Aß42 peptides extracted from various transgenic flies expressing Aß42 with an N-terminal signal peptide ran at the same position as the one from flies expressing Aß42 without signal peptide and the synthetic Aß42 (arrow). B) Protein extract from UAS-AßS7/+; GMR-Gal4/+ flies was analyzed by mass spectrometry. One peptide was detected and the molecular weight corresponds to Aß42 without signal peptide.

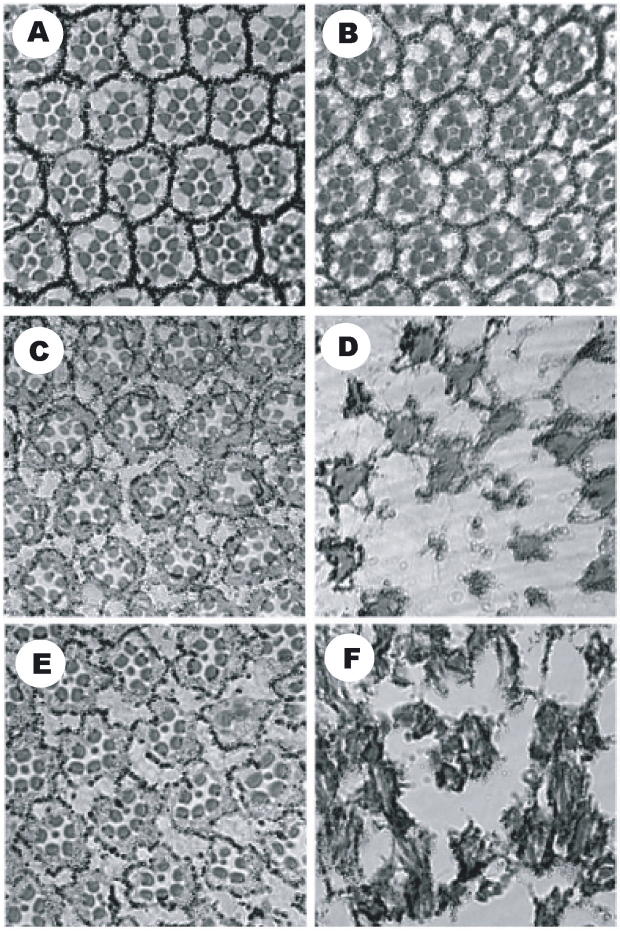


Fig 2. Aß42 mediated phenotypes correlate with age and expression levels

Aß42-mediated eye distortion. Aß-dependent eye phenotypes were worse in 30-day-old flies (D) than in 1-day-old flies (C). Control flies containing only GMR-Gal4 did not show such effects in both 1-day-old flies (A) and 30-day-old flies (B). (E) and (F) show eye sections of 5-day-old flies expressing Aß-moderately (UAS-AßS3/+; GMR-Gal4/+), or at high level (UAS-AßS7/+; GMR-Gal4/+).

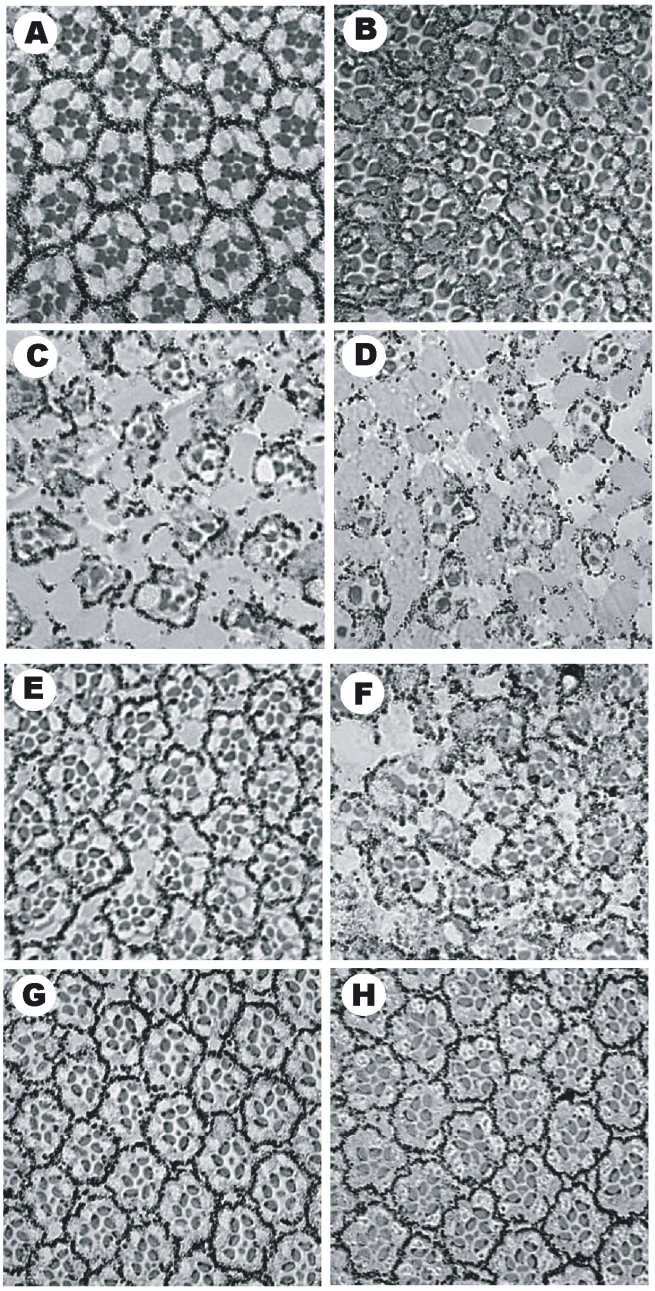
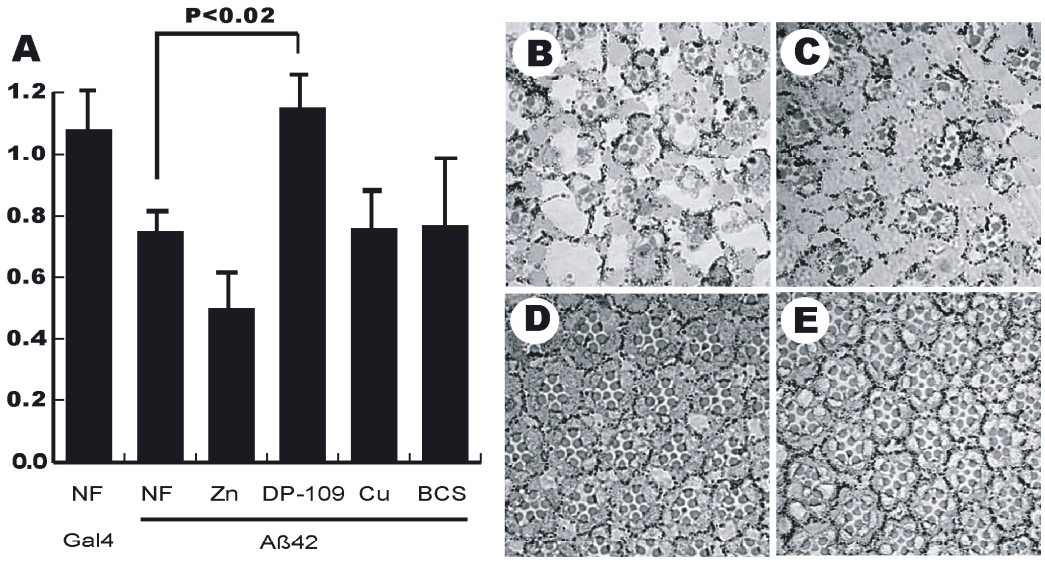


Fig 3. Zinc or copper load exacerbate eye distortion

Eye sections are shown of control flies (GMR-Gal4/+) grown on standard food (A) or UAS-AßS3/+; GMR-Gal4/+ flies grown on standard food (B), on 500 uM Cu (C) or on 4 mM Zn (D). Flies expressing wild type Aß (E, F) or Aß with 3His->Arg mutations (G, H) were treated with standard food (E, G) or food containing 4 mM Zn (F, H).



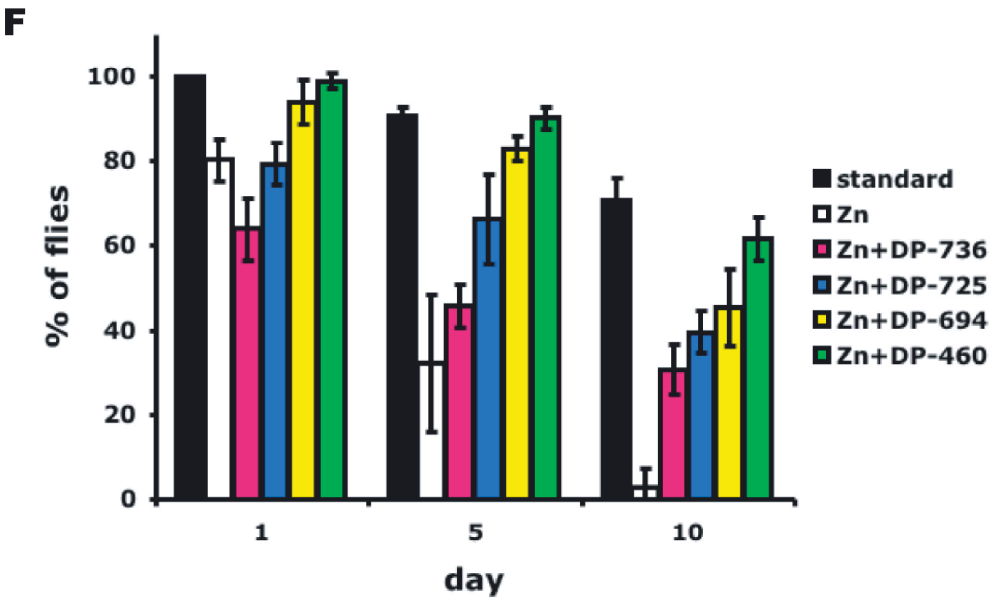


Fig 4. Metal chelaters improve Aß42-mediated phenotypes

(A) Survival assay using control flies and Aß expressing flies. Ubiquitous expression of Aß42 via actin-Gal4 led to a decrease in survival rate. The decline of survival can be restored by supplementation with the Cu/Zn chelator DP-109 but not by supplementation with BCS chelator. (B)-(D) Eye sections of UAS-AßS3/+; GMR-Gal4/+ flies raised on food containing 500 uM Cu (B) or 4 mM Zn (C), on food containing both 500 uM Cu and 250 uM BCS (D) or both 4 mM Zn and 100 uM DP-460 (E). (F) A climbing assay was used to monitor the function of UAS-AßS7/elav-Gal4 flies on standard food, or food containing 4 mM Zn or food containing 4 mM Zn in combination with various MACs (100 uM).

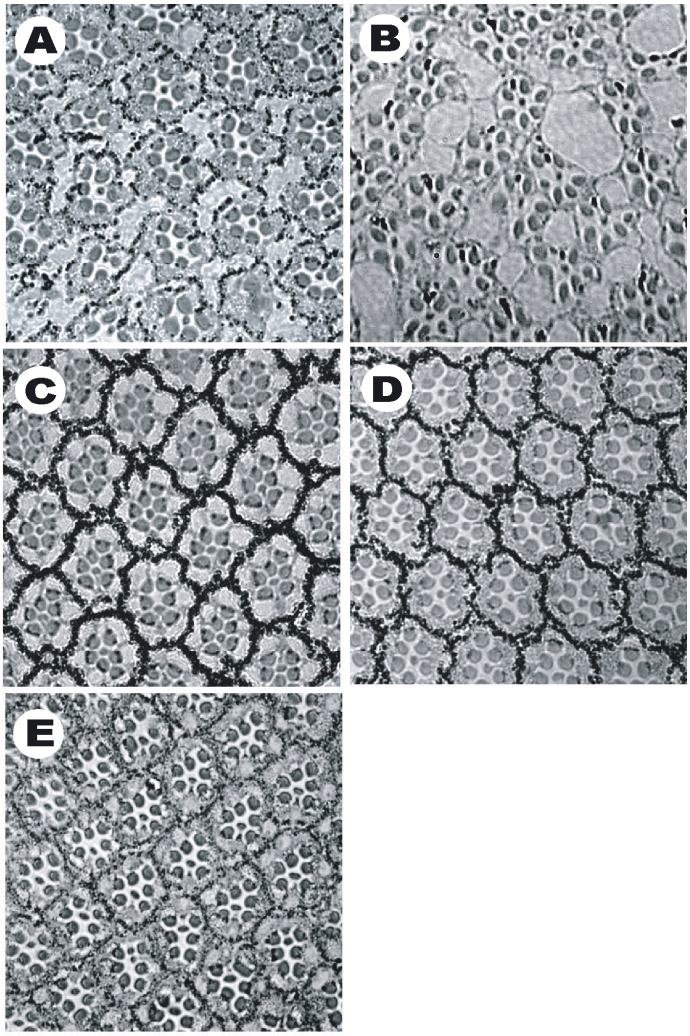


Fig 5. MTF-1 and metallothioneins ameliorate Aß42-mediated eye degeneration
Shown are eye section pictures of flies expressing Aß42 alone (A) or flies co-expressing Aß42 and dMTF-1 (C), MtnA (D), hMT3 (E). (B) Aß42 is expressed in flies lacking dMTF-1.

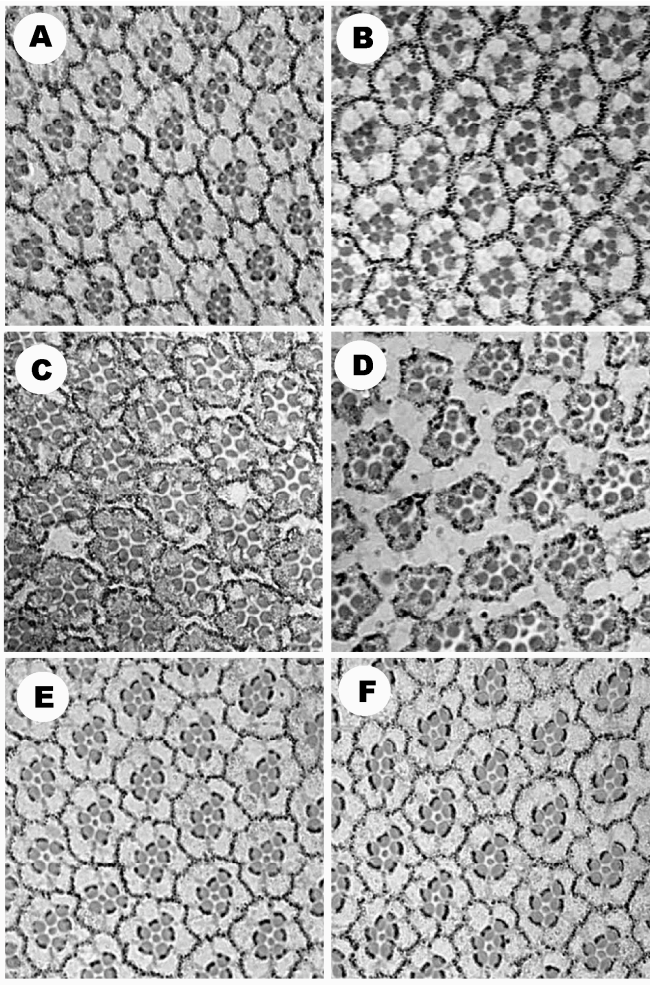


Fig 6. Aß-expressing flies, but not control flies, are hypersensitive to H_2O_2
Control flies (GMR-Gal4/+) displayed no obvious difference between standard food (A) and food containing 0.025% H_2O_2 (B). However, flies expressing Aß42 treated with 0.025% H_2O_2 (D) showed stronger eye degeneration than those grown on standard food (C). By contrast, expression of GCLc (E) or GCLm (F) in Aß42 expressing flies had beneficial effects.

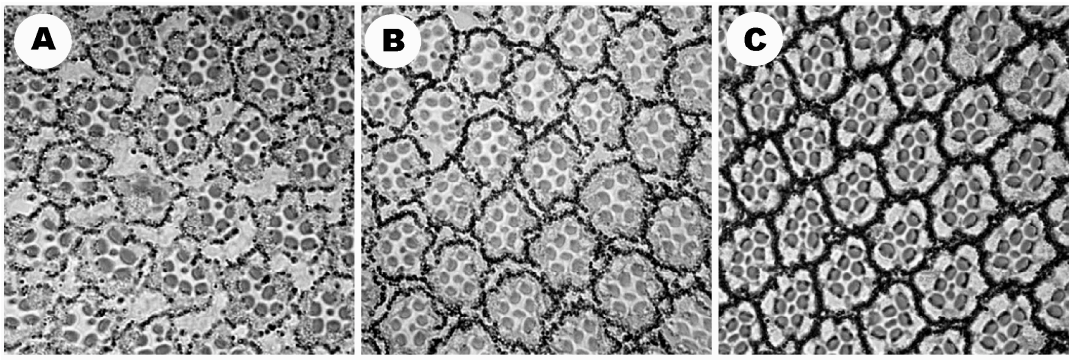


Fig 7. Aß42 induces cell death mainly via apoptosis

When Aß42 expression was combined with a deletion DfH99 (B) or p35 expression (C), the extent of cell death in the eye was lesser comparing to cell death in the eye that expressed Aß42 alone (A).

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